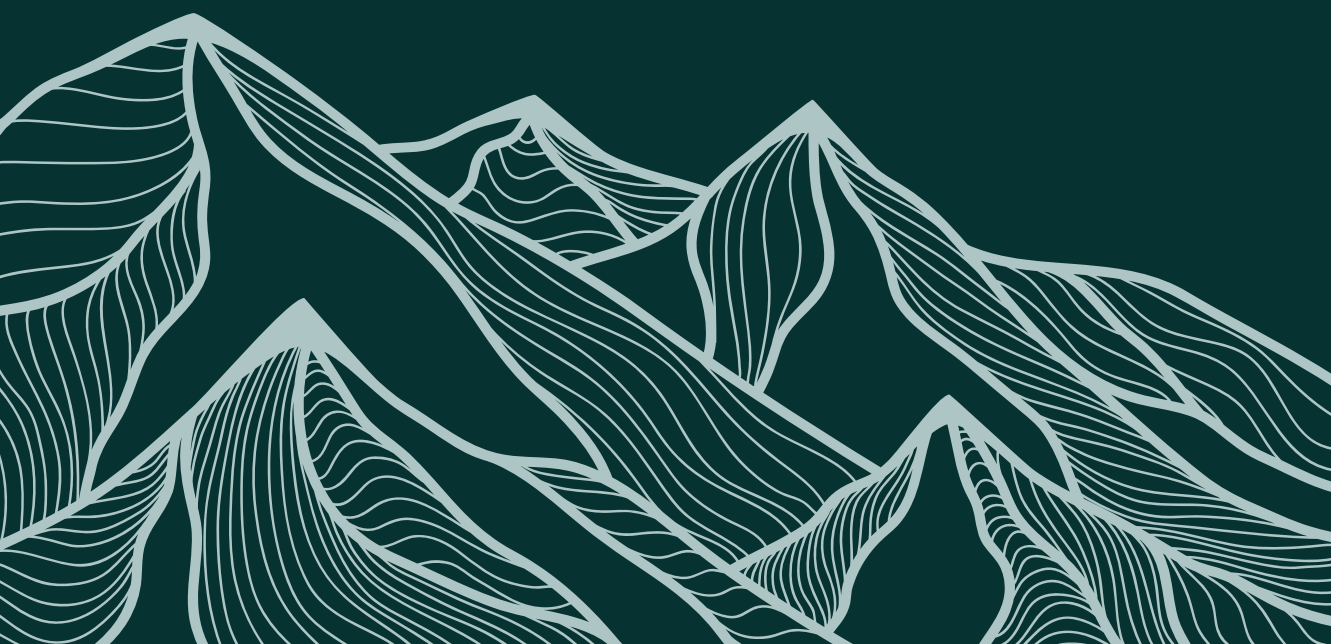
Immunotherapeutic strategies for the treatment of chondrosarcoma and the impact of resections around the shoulder

Sjoerd Pieter Nota



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PART I

Introduction and chondrosarcoma statistics

1

Chapter 1

General introduction and outline of the thesis

INTRODUCTION

Chondrosarcoma introduction

Chondrosarcoma is the second most common primary malignant bone tumor, representing roughly 25% of the primary malignant osseous neoplasms (1, 2). Chondrosarcoma are characterized by the production of chondroid matrix without the production of malignant osteoid.

Conventional central chondrosarcoma is the most frequent subtype making up approximately 90% of the cases characterized by malignant cells growing from the intramedullary cavity of bone. Peripheral chondrosarcoma is central chondrosarcoma's anatomical counterpart, developing from a pre-existing osteochondroma, situated on the outside of the bone. The peripheral chondrosarcoma affects younger patients and have a better prognosis when compared to central chondrosarcoma (3).

The dedifferentiated chondrosarcoma subtype consists of a well-differentiated chondrosarcoma (or enchondroma) juxtaposed by a high-grade poorly differentiated component (4), (Figure 1). These tumors are aggressive, and they metastasize early with a corresponding poor prognosis (5).

Patients presenting with high-grade chondrosarcoma typically show a long history of progressive pain and a substantial portion presents with a pathological fracture (6).

In the past, chondrosarcoma was graded on a scale from 1 to 3 determined by its cellularity, nuclei size, miotic activity and increased staining of the nuclei. The clinical behavior of chondrosarcoma is closely associated with this histologic grade. Low-grade chondrosarcoma (grade 1) is slow growing and have a low potential to metastasize. In contrast, high-grade chondrosarcoma (grade 2 and 3) and dedifferentiated chondrosarcoma are aggressive tumors with a poor prognosis and high risk of metastasis (7-9), (Figure 1).

The research in this thesis was performed while this classification was prevailing. Since 2020, a new classification is used after the publication of the new "WHO Classification of Tumours of Soft Tissue and Bone Tumours" (10). The term "Chondrosarcoma grade 1", now is only referring to lesions of the axial skeleton, including the flat bones, because these are behaving more aggressive than their counterparts in the appendicular skeleton, which are now called "Atypical Chondroid Tumours (ACT)".

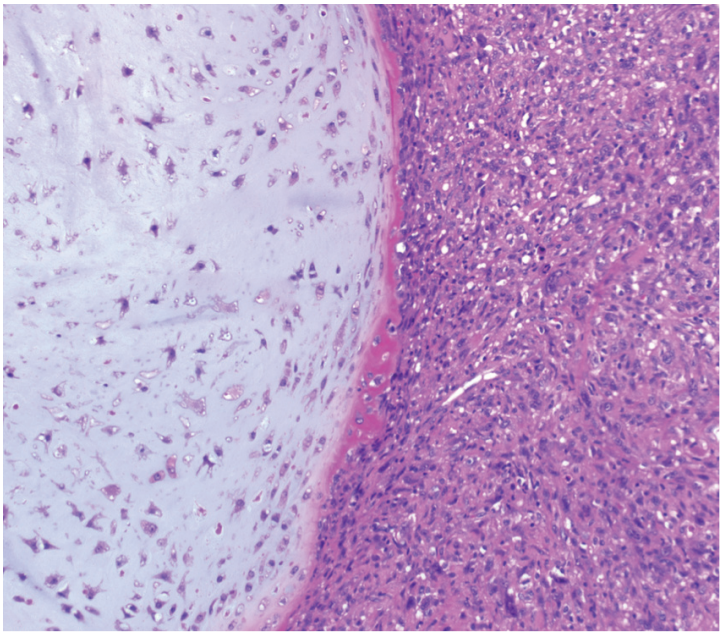


Figure 1. Hematoxylin and Eosin stained dedifferentiated chondrosarcoma showing a well-differentiated component (left) juxtaposed by a high-grade poorly differentiated component (right).

Chondrosarcoma treatment – tumor control and immunotherapy

Since conventional radiation therapy and chemotherapy have limited effect on conventional and dedifferentiated chondrosarcoma (11, 12), surgical resection remains the primary treatment. Except for Atypical Chondroid Tumorus (ACT's) in the appendicular skeleton, which can be treated with an extended intralesional curettage (13, 14), wide excision is the treatment of choice pursuing negative surgical margins (15). However, the oncological outcome is poor in high-grade tumors, locally recurrent disease and in disseminated disease (5, 16).

There is a need for effective systemic therapies for high-grade chondrosarcoma. In addition to an improved oncological outcome effective systemic therapies may also improve the quality of life for patients treated non-surgically, when tumor resection leads to unacceptable morbidity and in disseminated disease. In recent years major progress has been made in the treatment of different cancers by immunotherapeutic strategies (17-19). In different malignant diseases, promising clinical responses have been published (17, 18, 20-22). This convincing evidence has stimulated our interest in the development and application of immunotherapeutic therapies in chondrosarcoma.

The objective of immunotherapy is influencing a tumor antigen-specific immune response with the intention to eliminate cancer cells. All elements of the host's acquired and innate immune system participate in this intended tumor antigen-specific immune response.

An effective immune response is highly depended on the induction and functional activity of cytotoxic CD8+ T lymphocytes. The cytotoxic CD8+ T lymphocytes recognize tumor antigens expressed by tumor cells. These tumor antigens are processed and presented on the tumor cell surface by HLA class I antigen processing machinery (APM). This immune surveillance is continuously recognizing and eliminating nascent malignant cells (23).

Cancer cells have developed several escape mechanisms to avoid immune surveillance averting an immune reaction. In particular, defects in the APM, which include defects in HLA class I subunits (HLA class I heavy chain and beta-2 microglobulin) and in HLA class II molecules, cause tumors to evade the immune system (24-26). Generally these defects in HLA class I are not structural but a decreased expression which may be induced with certain methods such as radiation (27).

More recently studies have been focusing on immune checkpoint molecules. Normally these molecules help in preventing inappropriate targeting of normal cells by the host's immune system. However, cancer cells have been using these molecules to prevent an effective immune response by tumor infiltrating lymphocytes (28). B7-H3 and PD-1/PD-L1 are such immune checkpoint molecules currently being investigated as target in immunotherapy in several human cancers (29-32), (Figure 2).

The immune checkpoint molecule B7-H3 has limited expression on healthy tissues (33) and has most commonly been associated with the inhibition of cytotoxic CD8+ T lymphocytes (34). PD-L1, is expressed on both normal and cancer cells and releases inhibitory signals upon its interaction with its receptor PD-1 on T lymphocytes, hereby weakening their reactivity (29, 35).

Functional HLA I antigen expression is necessary to effectuate an immune response following checkpoint blockade inhibition and therefore not all tumors are suitable for these strategies. However, there are different immunotherapeutic approaches that function independently of HLA I expression. A treatment option that is independent of HLA I antigen expression is chimeric antigen receptor (CAR) T cell therapy. In this therapy T cells are genetically engineered to express synthetic receptors that recognize and eliminate cells expressing a specific target antigen (36, 37). This treatment has been proven effective in several other (liquid) cancers such as leukemia and lymphoma (38-40).

In this thesis we chose to investigate chondroitin sulfate proteoglycan 4 (CSPG4) as an additional immunotherapeutic target for CAR T cell therapy. CSPG4 is a cell surface proteoglycan that is expressed in chemically induced rat chondrosarcoma, later to be shown to share 100% homology with human CSPG4 (41-43). CSPG4 is highly expressed across several types of human cancers. Since it has limited expression in healthy tissues, it is an attractive target for antibody-based cancer immunotherapy. CSPG4 specific monoclonal antibodies as well as adoptive cell transfer therapies targeting CSPG4 have been effective in pre-clinical models by inhibition of tumor growth (43-48). The immunotherapeutic significance of CSPG4 in chondrosarcoma has not yet been explored.

In this thesis we will explore different immunotherapeutic strategies in chondrosarcoma.

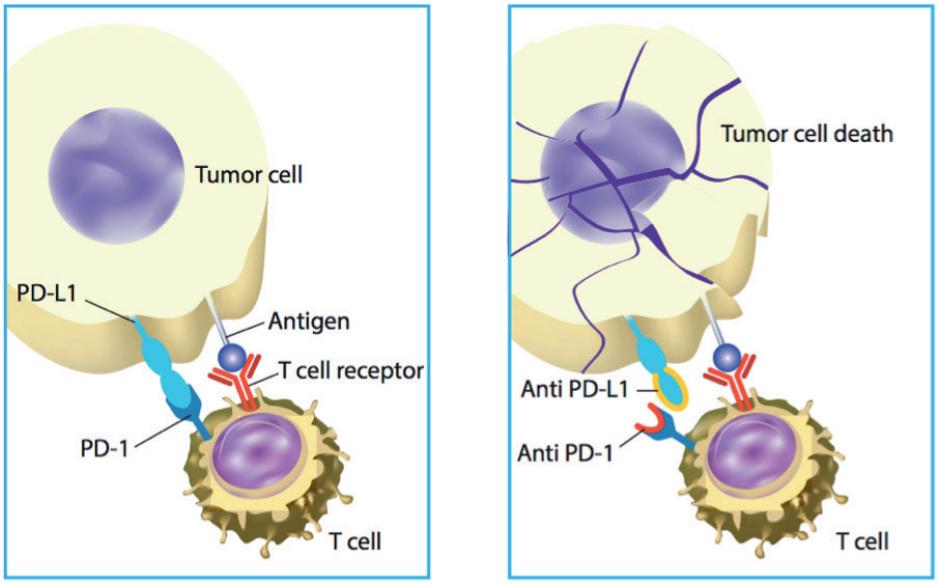


Figure 2. The left image showing to absence of an effective tumor infiltrating immune response, caused by the inhibitory checkpoint molecules PD-1/PD-L1. The right image showing the targeting of these immune checkpoint molecules, by anti-PD-1/PD-L1, leading to an adequate immune response.

Chondrosarcoma treatment - oncological resections around the shoulder.

Despite of the promising future systemic therapies, until now resection of chondrosarcoma, with adequate margins, remains the only curative option.

By pursuing negative surgical margins around the shoulder, the removal of crucial structures may be inevitable. This may imply partial resection of important structures such as the deltoid muscle, rotator cuff and axillary nerve. In addition, due to the associated bone loss after an adequate resection of the proximal humerus, glenoid and scapula, surgical reconstruction may be challenging.

In this thesis we investigate the impact of oncological resections of the shoulder since this is one of the most common locations for both conventional and dedifferentiated chondrosarcoma (5, 49).

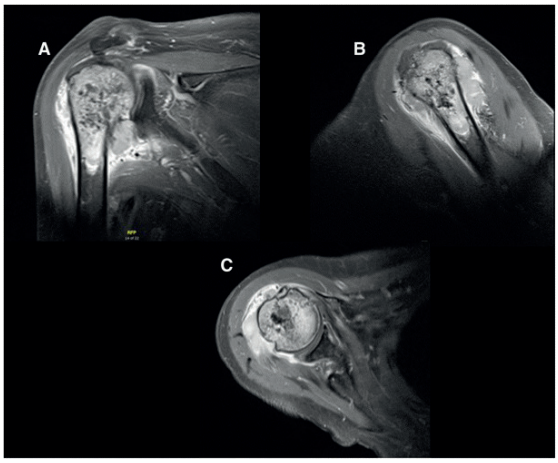
Following the resection of the proximal humerus several reconstructive options are in use today. There is no consensus about the preferred reconstruction method, allowing a functional glenohumeral joint. The main treatment options are: endoprostheses, osteoarticular allografts, and allograft-prosthesis composites.

Traditionally it is assumed that osteoarticular allografts have a higher rate of fractures, non-unions and infection while having the advantage of bone preservation, and soft tissue reattachment possibilities. Endoprotheses are thought to be at higher risk for subluxation, dislocation, proximal migration and limited abduction while benefitting from the rigidity and robustness of the prothesis.

The allograft-prosthesis composites reconstruction may have the combined advantages of the previous mentioned reconstructions but might combine some of the drawbacks as well, (Figure 3).

There are no randomized trials comparing these 3 different reconstructions with regard to the functional outcome, implant survivorship, or complications. The majority of studies evaluating function after oncological resection of the shoulder gather data from a physician's standpoint instead of reporting patient-reported outcomes.

In this thesis we investigate the functional differences between the 3 reconstructions as well as the implant survival and complication rates. In addition, we determined the functional and oncological outcome for patients treated operatively for scapular or clavicular chondrosarcoma.



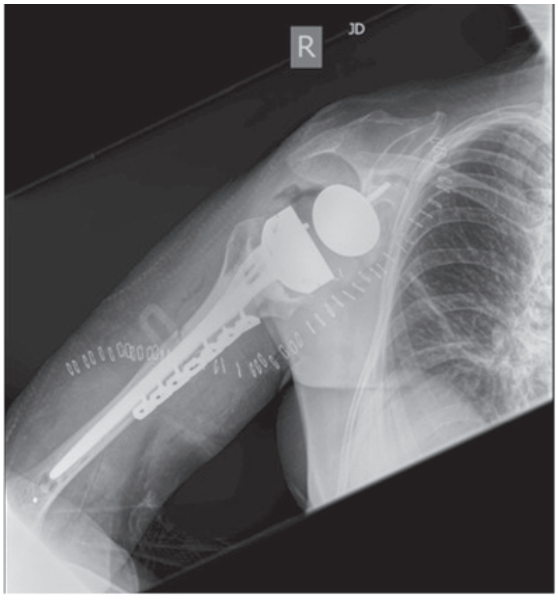


Figure 3A. MRI of the right shoulder showing a chondrosarcoma (T1 fat-suppressed post-contrast images). **Figure 3B.** Allograft-prosthesis composite reconstruction of the right proximal humerus.

(From: S. A. Lozano-Calderon & Neal Chen (2015) Proximal humerus allograft prosthetic composites: technique, outcomes, and pearls and pitfalls. Curr Rev Musculoskelet Med. 2015 Dec; 8(4): 324-333.)

OUTLINE OF THE THESIS

This thesis is divided in 4 parts, starting with **Part I** providing the general introduction and outline of the thesis, followed by an overview of the conventional prognostic factors and survival statistics of chondrosarcoma. In **Part II** the possible immunotherapeutic strategies in the treatment of chondrosarcoma are described. **Part III** presents the functional and oncological outcomes after tumor resections around the shoulder and **Part IV** provides a summary, discussion and future recommendations.

We performed a systematic review in **Chapter 2,** including 13 studies containing 1114 patients, with the aim to identify independent predictive factors and survival statistics for both conventional central chondrosarcoma and dedifferentiated central chondrosarcoma. This chapter pointed out that there is a need for studies identifying treatment options especially in high-grade and dedifferentiated chondrosarcoma.

Therefore, in **Chapter 3** the aim was to characterize the chondrosarcoma tumor infiltration by immune cells as well as the expression of immunological relevant molecules in chondrosarcoma specimens with immunohistochemistry staining techniques, in our self-developed tissue microarray. Hereby we aimed to contribute to the understanding of the role of immunological events in the pathogenesis of chondrosarcoma and to the design of immunotherapeutic strategies.

In **Chapter 4,** the goal was to assess the prevalence of CSPG4 in chondrosarcoma and to assess the efficacy of CSPG4 specific CAR T cells in lysing chondrosarcoma cells *in vitro* in 2 chondrosarcoma cell lines.

However, since surgery is the still the primary treatment of chondrosarcoma, we also investigated the impact of oncological resections. We decided to focus on resections around the shoulder since this is a commonly affected area for chondrosarcoma.

In **Chapter 5,** we looked at the best way of reconstructing the proximal humerus after its resection. We conducted a systematic review including 29 studies containing 693 patients. This included reconstructions with endoprostheses, osteoarticular allografts and allograft-prosthesis composites, aiming to evaluate which surgical reconstruction offers the best functional outcome, longest implant survival and lowest complication rate.

This was followed by **Chapter 6** where we decided to investigate a cohort of 150 patients in 2 hospitals who underwent a wide resection of the proximal humerus followed by the earlier mentioned oncologic reconstructions (endoprostheses, osteoarticular allografts and allograft-prosthesis composites). Their functional outcomes were collected prospectively and we retrospectively assessed complications

and implant survival with the goal to identify differences in these parameters between reconstructional methods.

Chapter 7 evaluates the functional and oncological outcome of 20 patients that were diagnosed with a primary chondrosarcoma of the scapula or clavicle. The surviving patients were functionally assessed and we longitudinally tracked with the aim to assess patients' function and oncological outcome. In **Chapter 8** we give a summary, and finally in **Chapter 9** we provide a discussion and a rationale for future studies.

AIMS OF THE THESIS

Part I

- Characterize the survival characteristics for conventional and dedifferentiated chondrosarcoma.
- Identify independent predictive factors for conventional and dedifferentiated chondrosarcoma.

Part II

- Identify tumor infiltration lymphocytes in conventional and dedifferentiated chondrosarcoma.
- Specify defects in HLA class I antigen processing machinery in conventional and dedifferentiated chondrosarcoma.
- Investigate the presence of the immune checkpoint molecules B7-H3 and PD-1/PD-L1 in conventional and dedifferentiated chondrosarcoma.
- Assess the expression of CSPG4 in conventional and dedifferentiated chondrosarcoma.
- Determine the effectiveness of CSPG4-specific CAR T cell therapy in eliminating chondrosarcoma cells *in vitro*.

Part III

- Identify which treatment option has the best functional result comparing: endoprostheses, osteoarticular allografts and allograft-prosthesis composites.
- Determine which of these treatment options have the longest implant survival and the lowest complication rate.
- Characterize the functional and oncological outcome for patients treated operatively for scapular or clavicular chondrosarcoma.

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2

Chapter 2

The Identification of Prognostic Factors and Survival Statistics of Conventional Central Chondrosarcoma.

Sarcoma. 2015

S.P.F.T. Nota, Y. Braun, J.H. Schwab, C.N. van Dijk, and J.A.M. Bramer

ABSTRACT

Introduction

Chondrosarcomas are malignant bone tumors that are characterized by the production of chondroid tissue. Since radiation therapy and chemotherapy have limited effect on chondrosarcoma, treatment of most patients depends on surgical resection. We conducted this study to identify independent predictive factors and survival characteristics for conventional central chondrosarcoma and dedifferentiated central chondrosarcoma.

Methods

A systematic literature review was performed in September 2014 using the Pubmed, Embase, and Cochrane databases. Subsequent to a beforehand-composed selection procedure we included 13 studies, comprising a total of 1114 patients.

Results

The prognosis of central chondrosarcoma is generally good for the histologically low-grade tumors. Prognosis for the high-grade chondrosarcoma and the dedifferentiated chondrosarcoma is poor with lower survival rates. Poor prognostic factors in conventional chondrosarcoma for overall survival are high-grade tumors and axial/pelvic tumor location. In dedifferentiated chondrosarcoma the percentage of dedifferentiated component has significant influence on disease-free survival.

Conclusion

Despite the fact that there are multiple prognostic factors identified, as shown in this study, there is a need for prospective and comparative studies. The resulting knowledge about prognostic factors and survival can give direction in the development of better therapies. This could eventually lead to an evidence-based foundation for treating chondrosarcoma patients.

INTRODUCTION

Chondrosarcomas are malignant bone tumors that can be characterized by the production of chondroid tissue [1]. This heterogeneous group of tumors occupy about a quarter of all the primary malignant osseous neoplasms of the bone [2]. Chondrosarcomas are the most common occurring primary sarcoma of the bone after osteosarcoma [2, 3]. The clinical behavior and prognosis of these tumors depend on many variables of which tumor grade is one of the most important; high-grade tumors have a worse prognosis compared to low-grade tumors [4, 5]. This poor prognosis can partially be explained by the high tendency to metastasize. About three-quarters of all chondrosarcomas consist of conventional central chondrosarcoma. These central chondrosarcomas have the outgrowth of the sarcomatous tumor in the intramedullary cavity in common. The central chondrosarcoma's anatomical counterpart is the peripheral chondrosarcoma. These specific chondrosarcomas develop from a preexisting osteochondroma and are situated on the outside of the cortex of the bone. The peripheral chondrosarcoma tumors have a better prognosis when compared to the central chondrosarcoma and tend to affect younger patients [6].

Radiation therapy and chemotherapy have limited to arguably no effect on conventional chondrosarcoma [7, 8]. There are rarer chondrosarcoma subtypes that are more responsive to chemotherapy and/or radiation therapy [9]. The vast majority of chondrosarcoma patients solely depended on the surgical treatment by tumor resection. Chemotherapy might have a role in dedifferentiated chondrosarcoma [10, 11] although the positive effect is not consistently reported in literature [11–13].

Identification of prognostic factors and knowledge about survival are important. For patients this knowledge can provide insight into their future perspective and it may provide guidance in the decision-making concerning treatment. Physicians can use the prognostic and survival information as a tool to select the optimal treatment strategy and inform patients. To direct efforts in the development of new therapeutic strategies the identification of proven prognostic factors of central chondrosarcoma is important, especially since the treatment options are limited. We conducted this systematic review with the aim of identifying independent predictive factors and survival characteristics for both conventional central chondrosarcoma and dedifferentiated central chondrosarcoma.

MATERIALS AND METHODS

This systematic review was registered on PROSPERO prior to data extraction (registration number: CRD42014008961). The MOOSE checklist for meta-analysis

of observational studies in epidemiology study was applied for the evaluation of meta- analysis and observational studies [14].

2.1. Search Strategy

We searched Pubmed, Embase, and the Cochrane database for title and abstract, without any limits on September 9, 2014, using the following search terms: ((“chondrosarcoma* ” OR “chondroid sarcoma” OR “chondroid sarcomas”) AND “prognos*”) OR ((“chondrosarcoma* ” OR “chondroid sarcoma” OR “chondroid sarcomas”) AND “surviv*”) resulting in a total of 2253 publications.

2.2. Study Selection, Data Extraction, and Critical Appraisal

Two reviewers (Sjoerd P. F. T. Nota, Yvonne Braun) independently screened all the studies’ titles and abstracts and retrieved the full-text manuscripts for the articles that met our inclusion criteria. If consensus was not reached between the two reviewers, a third reviewer (Jos A. M. Bramer) was consulted. We included all articles focusing on any prognostic factors and/or survival statistics on all grades (including dedifferentiated chondrosarcoma) of primary central chondrosarcoma of the bone.

We excluded congress proceedings, letter to the editors, cohorts that were not independently identifiable, all studies published in a different language than English, and studies published before 1980. In addition we excluded case-reports and case-series with less than 10 patients. Furthermore we excluded papers reporting on surgical procedures and studies focusing solely on metastasis. Finally we excluded all papers that did not clearly distinguish between central and peripheral chondrosarcoma and reviews were excluded as well. After applying our exclusion criteria on the title and abstract 274 papers remained for full-text screening. The quality of the data was assessed by application of predetermined critical appraisal criteria by two independent researchers. Lack of consensus was solved again as described above. The criteria assessed were as follows: study participation, study attrition, prognostic factor measurement, outcome measurement, confounding measurement, analysis performed, population included, the time of follow-up, the level of evidence, the presence of a disclosure statement, and the presence of a baseline characteristics table (Appendix).

2.3. Outcome Measurements

We extracted the data of the following variables from the selected studies: author/year, type of study, mean age, sex distribution, mean duration of follow-up, primary tumors only, metastasis at presentation, grading method, tumor grade, anatomical location, overall survival, and 5- and 10-year survival per grade. In addition we registered the disease-free survival, the percentage of patients with no evidence of disease, and the percentage of patients with no evidence of disease after tumor

relapse. Furthermore we looked at the percentage of patients alive with disease and dead of disease and the percentage of patients that died of a different cause. We also looked at the local recurrence rate, the time to local recurrence, metastasis rate and time to metastasis, and the use of chemo- and radiation therapy. Finally to account for the homogeneity of the treatment of the patients in the studies we also reported the status of the surgical margins of the included subjects.

2.4. Analysis

To prevent reporting biased results due to the high quantity of cohort studies and case-series and potential overlap of patients' population we choose to only report our results narratively and did not attempt to merge results and do additional analyses.

2.5. Prognostic Factors and Survival Statistics

In this review we will narratively summarize the prognostic factors and survival statistics reported in our selected studies.

2.6. Study Characteristic

After screening the full-text articles we included 13 studies that met our inclusion criteria for this review [10, 15–26]. The 13 studies included were based on retrospective evidence. All studies reported clearly the dates of researched period, the patient sample, and the point of the course of the disease. Nine out of the 13 studies (69%) reported a sufficient long follow-up (more than 1 year) and explained the reason of patients being lost to follow-up. Four studies (31%) did not report these factors and may therefore be subject to more selection bias (see the appendix).

2.7. Study Population

The 13 included studies comprised the data of 1114 patients, although population overlap is likely since multiple studies are performed in the same institution. In the studies where we could determine the age the average age of the patients ranged from 35 to 59 years and the percentage of males ranged from 42% to 79% with only 1 study reporting more females in the cohort. The mean follow-up ranged from at least more than 2 years to 13 years. The individual follow-up ranged from a minimum of 0 years to a maximum of 26 years (Table 1).

Not all studies mentioned the fact if only primary tumors and if recurrences were excluded or did included patient with such tumors (Table 2). There were a wide variety of tumor grades in the included studies. Three studies focused on central dedifferentiated chondrosarcoma and 1 study focused on grade 2 chondrosarcoma only; all other studies included patients with a variety of different tumor grades. The localizations of the tumors comprise the entire skeleton throughout the different studies (Table 2).

In 10 out of the 13 studies the surgical margin status was determined showing a wide range in the percentages of patients having wide and radical resection (Table 6).

The additional use of chemotherapy and radiotherapy is only registered in, respectively, 9 out of 13 (69%) and 6 out of 13 (46%) studies. Chemotherapy is used in 6 out of the 9 (67%) studies where chemotherapy is mentioned. Radiotherapy is used in 4 out of the 6 (67%) studies where its use is mentioned (Table 5).

Table 1: Demographic patient and study characteristics of the included studies

Study	Study design	Patients	Mean age (range)	Male	Follow-up (range)
		(Number)	(Years)	(%)	(Years)
Andreou et al., 2011	R	115	47 (14-79)	61%	12 (5-24)
Angelini et al., 2012	R	296	50 (13-88)	57%	7 (1.6-20)
Briccoli et al., 2002	R	14	.	.	5.8 (0-19)
Cho et al., 2011	R	32	.	72%	9.2 (2.6-19)
De Camargo et al., 2010	R	46	43 (17-79)	54%	8.3 (2.7-26)
Donati et al., 2010	R	31	35 (13-67)	42%	13 (5.5-25)
Donati et al., 2005	R	63	.	.	.
Gitelis et al., 1981	R	69	44 (14-78)	68%	>5 year
Mavrogenis et al., 2013	R	119	.	.	>2 year
Mitchell et al., 2000	R	14	57 (37-79)	79%	4.7 (1.7-7.5)+
Ozaki et al., 1996	R	21	51 (25-71)	67%	12 (5-22)
Staals et al., 2006	R	123*	59 (24-83)	54%	2.8 (0-17)
Van Maldegem et al., 2014	R	171	53 (17-90)	63%	.

* 110 patient with actual follow-up data, R=retrospective, + surviving patients

RESULTS

3.1. Survival: General

Overall survival ranged from 21% to 100% at the time of follow-up depending on the specific study. Five- and 10-year survival ranged from 2% to 100% and 32% to 85%, respectively (Table 3). Disease-free survival ranged from 30% to 89% and the local recurrence rate ranged from 6.2% to 35%. In the 5 studies reporting the metastasis rate the rate ranges from 0% to 38% (Table 4).

3.2. Survival: Grades 1, 2, and 3 and Dedifferentiated Chondrosarcoma

The reported 5-year survival for grade 1 chondrosarcoma ranged from 82% to 99%. The 10-year survival ranged from 89% to 95%. The 5-year survival for grade 2 chondrosarcoma ranged from 63% to 92%. The 10-year survival ranged from 58% to 86%. The 5-year survival for grade 3 chondrosarcoma ranged from 0% to 77%. The lowest (0%) survival was displayed in a study looking at a very small subgroup of patients treated with an intralesional resection. The 10-year survival ranged from 0% to 55%. The 5-year survival for dedifferentiated chondrosarcoma was 24% as reported in 1 study (Table 3).

3.3. Prognostic Factors.

In 7 of the included studies prognostic factors for overall survival were reported. Cho et al. found no difference in event-free survival between curettage in combination with subsequent treatment versus standard treatment of wide excision ($p = 0.16$) in their cohort of grade 2 chondrosarcoma of the extremities [18]. Donati et al. compared survival of central with peripheral chondrosarcoma and found a difference in survival in their cohort of pelvis tumors ($p = 0.00093$) as did Gitelis et al. at 5-year ($p < 0.001$) and 10-year ($p < 0.001$) as well as in total disease-free survival ($p < 0.005$) between central and peripheral chondrosarcoma [21, 22].

Andreou et al., Angelini et al., and Staals et al. investigated multiple potential prognostic factors [15, 16, 25] as summarized in Table 7. The main significant poor prognostic factors Andreou et al. and/or Angelini et al. reported were larger tumor volume, higher grade and distant metastasis, and a worse prognosis for axial located tumors (including the pelvis) compared to in the extremity located tumors. Worst prognosis for a pathologic fracture and supportive care in comparison with multidisciplinary treatment (chemotherapy, radiotherapy, and further surgery) were reported as well. Staals et al. most prominent findings were the significant impact of Stage 3 lesions versus, respectively, Stages 2a and 2b lesions and the poor prognostic value of a higher percentage of dedifferentiated component within the tumor [25]. van Maldegem et al. show in unresectable chondrosarcoma a survival benefit for the use of chemotherapy compared to not using systemic treatment. When interpreting these results, the large heterogeneity in the treatment groups should be accounted for. In addition they show significant impact of solely unresectable disease compared to unresectable disease in combination with the presence of metastasis. Finally they showed that an age younger than 40 and grade 2 tumors have a better survival [26] (Table 7).

Table 2: Oncologic patient and study characteristics of the included studies.

Study	Primary tumors only	Metastasis at presentation	Grading	Gade 1	Grade 2	Grade 3	Dediff.
Andreou et al., 2011	yes	no	Evans et al.	56 (49%)	41 (36%)	18 (15%)	0
Angelini et al., 2012	.	.	Lichtenstein and Jaffe	87 (29%)	162 (55%)	47 (16%)	0
Briccoli et al., 2002	no	yes	.	0	9 (64%)	4 (29%)	1 (7.1%)
Cho et al., 2011	no	no	Evans et al.	0	32 (100%)	0	0
De Camargo et al., 2010	yes	yes	Lichtenstein and Jaffe	23 (50%)	23 (50%)	0	0
Donati et al., 2010	no	no	Mirra et al. + Schiller er al.	31 (100%)	0	0	0
Donati et al., 2005	no	.	.	1 (1.6%)	44 (70%)	18 (29%)	0
Gitelis et al., 1981	.	.	.	9 (13%)	46 (67%)	14 (20%)	0
Mavrogenis et al., 2013	.	.	.	.	.	.	22 (18%)
Mitchell et al., 2000	.	.	.	0	0	0	14 (100%)
Ozaki et al., 1996	no	.	Evans et al.	11 (52%)	6 (29%)	4 (19%)	0
Staals et al., 2006	.	.	.	0	0	0	123 (100%)
Van Maldegem et al., 2014	no	yes	.	9	118	44	0

Study	Lower Extremity	Upper Extremity (incl. shoulder girdle)	Hand*	Pelvis girdle (incl. sarcrum)	Axial skeleton	Thorax	Other
Andreou et al., 2011	48 (42%)	20 (17%)	0	42 (37%)	5 (4.3%)	0	0
Angelini et al., 2012	134 (45%)	50 (17%)	4 (1.4%)	82 (28%)	10 (3.4%)	16 (5.4%)	0
Briccoli et al., 2002	0	0	0	0	0	14 (100%)	0
Cho et al., 2011	22 (69%)	7 (22%)	0	0	0	0	3 (9.4%)
De Camargo et al., 2010	25 (54%)	10 (22%)	4 (8.7%)	6 (13%)	1 (2.2%)	0	0
Donati et al., 2010	24 (77%)	7 (23%)	0	0	0	0	0
Donati et al., 2005	0	0	0	63 (100%)	0	0	0
Gitelis et al., 1981	.	.	.	.	.	.	.
Mavrogenis et al., 2013	0	0	0	119 (100%)	0	0	0
Mitchell et al., 2000	8 (57%)	2 (14%)	0	4 (29%)	0	0	0
Ozaki et al., 1996	6 (29%)	2 (9.5%)	0	13 (62%)	0	0	0
Staals et al., 2006	67 (54%)	27 (22%)	0	28 (23%)	0	0	0
Van Maldegem et al., 2014	^	^	^	63 (37%)	9 (5.3%)	15 (8.8%)	^

* if specific mentioned, ^ no differentiation possible

Table 3: Oncologic outcome, survival

Study	Overall survival	5y survival	10y survival	Grade 1 5y survival	Grade 1 10y survival
Andreou et al., 2011	63%	72%	69%	89%	89%
Angelini et al., 2012	84%	92%	84%	99%	95%
Briccoli et al., 2002	86%	.	.	.	.
Cho et al., 2011	84%	.	85%^	.	.
De Camargo et al., 2010	94%^^	.	.	.	.
Donati et al., 2010	100%	100%	.	.	.
Donati et al., 2005	73%	.	.	.	.
Gitelis et al., 1981	.	49%	32%	.	.
Mavrogenis et al., 2013	.	80%*	65%*	.	.
Mitchell et al., 2000	21%	.	.	.	.
Ozaki et al., 1996	57%	.	.	82%	.
Staals et al., 2006	24%	24%	.	.	.
Van Maldegem et al., 2014	.	2%	.	.	.
Study	Grade 2 5y survival	Grade 2 10y survival	Grade 3 5y survival	Grade 3 10y survival	Dedifferentiated 5y survival
Andreou et al., 2011	63%	58%	39%	33%	.
Angelini et al., 2012	92%	86%	77%	55%	.
Briccoli et al., 2002	.	.	50%	.	.
Cho et al., 2011	.	85%^	.	.	.
De Camargo et al., 2010	.	.	.	.	.
Donati et al., 2010	.	.	.	.	.
Donati et al., 2005	.	.	.	.	.
Gitelis et al., 1981	.	.	.	.	.
Mavrogenis et al., 2013	.	.	.	.	.
Mitchell et al., 2000	.	.	.	.	.
Ozaki et al., 1996	67%	.	0%	.	.
Staals et al., 2006	.	.	.	.	24%
Van Maldegem et al., 2014	.	.	.	.	.

* extracted from Kaplan Meier curve, ^^43/46=93%, "+" of patients with enough FU,

^ discrepancy calculation and manuscript

Table 4: Oncologic outcome, survival

Study	Disease free survival	No evidence of disease	No evidence of disease after tumor relapse	Alive with disease with disease	Dead of disease	Dead of other cause
Andreou et al., 2011	63%	73 (63%)	0	0	38* (33%)	4 (3.5%)
Angelini et al., 2012	79%	201 (68%)	33 (11%)	15 (5.1%)	35 (12%)	12 (4.1%)
Briccoli et al., 2002	71%	10 (71%)	.	2 (14%)	1 (7.1%)	1 (7.1%)
Cho et al., 2011	75%	24 (75%)	2 (6.3%)	3 (9.4%)	5 (16%)	0
De Camargo et al., 2010	89%	.	.	6 (13%)	3 (7%)	0
Donati et al., 2010	.	29 (94%)	2 (6.5%)	0	0	0
Donati et al., 2005	.	.	.	.	.	.
Gitelis et al., 1981	30%	.	.	.	.	.
Mavrogenis et al., 2013	.	.	.	.	.	.
Mitchell et al., 2000	.	.	.	.	.	.
Ozaki et al., 1996	62%	4 (19%)	9 (43%)	0	7 (33%)	1 (4.8%)
Staals et al., 2006	.	.	.	.	84 (76%)	.
Van Maldegem et al., 2014	.	.	.	.	.	.

* including 6 treatment related deaths

Table 5

Study	Local recurrence	Time to local recurrence (months)	Metastasis	Time to metastasis Months	Chemo-therapy	Radiation
Andreou et al., 2011	38 (33%)	21 (2-96)	30 (26%)	27 (2-141)	Used	Used
Angelini et al., 2012	50 (17%)	.	41 (14%)	.	Not used	Not used
Briccoli et al., 2002	6 (43%)	.	.	.	Used	.
Cho et al., 2011	2 (6.2%)	.	10 (31%)	49 (7-181)*	.	.
De Camargo et al., 2010	16 (35%)	24 (9-46)	.	.	Not used	Not used
Donati et al., 2010	2 (6.5%)	31 (31-31)	0	.	.	.
Donati et al., 2005	15 (24%)	.	.	.	.	.
Gitelis et al., 1981	22 (32%)	.	26 (38%)	.	.	.
Mavrogenis et al., 2013	.	.	.	.	Used	.
Mitchell et al., 2000	.	.	.	.	Used	Used
Ozaki et al., 1996	.	.	.	.	Not used	Used
Staals et al., 2006	.	.	.	.	Used	.
Van Maldegem et al., 2014	.	.	.	.	Used	Used

* different numbers calculatable in paper

Table 6

Study	Inadequate surgical margins Enneking: intralesional or marginal	Wide and radical margin
Andreou et al., 2011	21 (18%)	94 (82%)
Angelini et al., 2012	74 (25%)	222 (75%)
Briccoli et al., 2002	3 (21%)	11 (79%)
Cho et al., 2011	7 (22%)	25 (78%)
De Camargo et al., 2010	25 (54%)	18 (39%)
Donati et al., 2010	17 (55%)	14 (45%)
Donati et al., 2005	17 (27%)	46 (73%)
Gitelis et al., 1981	37 (54%)	32 (46%)
Mavrogenis et al., 2013	.	.
Mitchell et al., 2000	.	.
Ozaki et al., 1996	21 (100%)	0
Staals et al., 2006	.	.
Van Maldegem et al., 2014	52 (30%)*	87 (51%)*

* initial surgery

Table 7: prognostic factors

Andreou et al.		**Angelini et al.**	
Overall survival		Overall survival	
Variable (Bivariate analysis)	**P-value**	**Variable (Bivariate analysis)**	**P-value**
Sex	p=0.6	G1: wide vs. intralesional	p=0.495
Age (higher)	p=0.04	G1: extremity vs. trunk	p=0.595
Extremity vs. axial + pelvis	p=0.002	G2: wide vs. intralesional	p=0.948
Tumor volume (0-100cc vs. >100cc)	p<0.001	G2: extremity vs. trunk	p=0.589
Grade tumor	p<0.001	G2: resect. vs. amputation	p=0.496
Local recurrences	p<0.001	G3: extremity vs. trunk	p=0.039
Distant metastasis	p<0.001	G3: resect. vs. amputation	p=0.051
Surgical margins	p=0.9	.	.
Type of surgery	.	.	.
low grade: ablative vs. limb-sparing	p=0.7	.	.
high grade: ablative vs. limb-sparing	p=0.1	.	.
Pathologic fracture	p=0.002	.	.
ACJCC	p<0.001	.	.
Multi disc. vs. support. care:	p=0.001	.	.
.	.	.	.
Variable (Multivariate analysis)	.	**Variable (Multivariate analysis)**	
High grade: RR=5	p<0.001	G3: resect. vs amputation	p=0.0943
Axial + pelvis: RR=2	p=0.04	G3: extr. vs. trunk	p=0.0889

Staals et al.		**Van Maldegem et al.**	
Disease free survival		Overall survival from the day of unresectability	
Variable (Bivariate analysis)	**P-value**	**Variable (Bivariate analysis)**	**P-value**
Gender	NS	Only local unresectable disease vs. local unresectable disease + metastasis	p=0.0014
Age	NS	Age (<40 years)	p=0.001
Duration of symptoms	NS	Grade II tumors	p=0.022
Lesion size	NS	Sex	NS
Anatomic location	NS	Site	NS
Stage 3 vs. Stage 2a	p=0.003	Resectable vs. non-resectable disease at primary diagnosis	NS
Stage 3 vs. Stage 2b	p<0.00005	Systemic treatment	p<0.0487
Stage 2a vs. Stage 2b	p=0.27	.	.
Histologic subtype, MFH vs. OS	p=0.046	.	.
Histologic subtype, MFH vs. fibr. sarc.	p=0.08	.	.
Histologic subtype, OS vs. fibr. sarc.	p=0.96	.	.
Grade 3DD vs. Grade 4DD	p=0.10	.	.
Percentage of DD component	p=0.0102	.	.
Percentage of DD component, >50% vs <50%	p<0.00005	.	.
Limb sparing vs. resection	p=0.08	.	.
Surgery vs. surgery + chemotherapy	p=0.88	.	.
Variable (Multivariate analysis, overall survial)	.	.	.
Percentage of DD component	p=0.0102	.	.

RR= Relative Risk, G=Grade, MFH= Malignant fibrohistocytoma, OS=Osteosarcoma, DD=dedifferentiated

DISCUSSION AND CONCLUSIONS

The results of our study show that the prognosis of central chondrosarcoma is fairly good for the low histological grade tumors with a 5- and 10-year survival of over 80%. High-grade chondrosarcoma and the highly lethal dedifferentiated chondrosarcoma have a poor prognosis with lower survival rates. The main negative prognostic factors for overall survival displayed in this review are a higher tumor grade and an axial/pelvis location of the tumor for the conventional chondrosarcoma. The percentage of dedifferentiated component within dedifferentiated chondrosarcoma has significant influence on disease-free survival of these tumors.

This review should be interpreted with its limitations in mind. First of all there are only limited studies in literature that describe solely central chondrosarcoma (or where the central chondrosarcomas are identifiable). The included studies are all retrospective and, even though we used strict inclusion criteria, have a large heterogeneity between patients and treatments. The heterogeneity in histologic type of grading used to evaluate the tumors, the variability in the use of chemo- and radiotherapy, and the differences in the presence of inadequate surgical margins might all have influenced our study's main outcomes. Second limitation is the likely overlap in patient population that can be explained by the centralization of care in large institutions due to the low incidence of primary orthopaedic tumors in general populations. This might introduce a bias and might amplify the experience of a single (experienced) center. Finally there is a large heterogeneity in the outcome measures, partially explained by differences in follow-up time, which makes the direct comparison and getting a general overview of the included studies challenging. This is, for example, displayed in the wide ranges in survival statistics. The grade 3 tumors have a range of 0–77% 5-year survival. Most likely this difference is caused by comparing a small subgroup of intralesional treated tumors with the results from a highly specialized center. Also significant interobserver variability in pathologists' histologic grading is known to be present in these types of tumors [27]. This might also directly influence the reported outcomes.

Remarkably in contrast to reports in literature on chondrosarcoma [28, 29], surgical margins were not identified as independent predictor of survival in this review. However, as stated by Andreou et al. as well, in multivariable analysis Lee et al. showed only a small effect of surgical margin status on survival and Fiorenza et al. were not able to determine the effect when accounting for confounders factors [15]. Caution is needed when interpreting these conclusions and their potential consequences in practice. Relative small retrospective studies with a large heterogeneity of patients might be the cause of the inability to identify, in oncology commonly accepted, prognostic factors such as wide surgical margins.

Our study points out that there is a need for prospective and comparative studies identifying factors and treatments influencing the survival of patients suffering from central chondrosarcoma. More evidence from high quality research might eventually lead to a more evidence-based foundation of treatments while preventing abundant exposure of patients to potentially harmful therapies such as radiation and chemotherapy. Further centralization of care for patients with relatively rare diseases would be desirable from a patient's point of view but might also generate opportunities for researchers to set up prospective and comparative studies. To improve survival in central chondrosarcoma patients, the high-grade chondrosarcoma and the dedifferentiated chondrosarcoma seem to be good candidates for future studies exploring better treatments options due to their poor prognosis.

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PART II

Immunotherapeutic leads in chondrosarcoma

3

Chapter 3

High TIL, HLA, and Immune Checkpoint Expression in Conventional High-Grade and Dedifferentiated Chondrosarcoma and Poor Clinical Course of the Disease.

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ABSTRACT

Purpose

The aim of this study was to characterize chondrosarcoma tumor infiltration by immune cells and the expression of immunologically relevant molecules. This information may contribute to our understanding of the role of immunological events in the pathogenesis of chondrosarcoma and to the rational design of immunotherapeutic strategies.

Patients and Methods

A tissue microarray (TMA) containing 52 conventional and 24 dedifferentiated chondrosarcoma specimens was analyzed by immunohistochemical staining for the expression of parameters associated with tumor antigen-specific immune responses, namely, CD4+ and CD8+ tumor infiltrating lymphocytes (TILs) and the expression of HLA class I heavy chain, beta-2 microglobulin (β2m), HLA class II and immune checkpoint molecules, B7-H3 and PD-1/PD-L1. The results were correlated with histopathological characteristics and the clinical course of the disease.

Results

CD8+ TILs were present in 21% of the conventional and 90% of the dedifferentiated chondrosarcoma tumors tested. B7-H3 was expressed in 69% of the conventional and 96% of the dedifferentiated chondrosarcoma tumors tested. PD-1 and PD-L1 were expressed 53% and 33% respectively of the dedifferentiated tumors tested. PD-L1 expression was associated with shorter time to metastasis.

Conclusion

The tumor infiltration by lymphocytes suggests that chondrosarcoma is immunogenic. Defects in HLA class I antigen and expression of the checkpoint molecules B7-H3 and PD-1/PD-L1 suggest that tumor cells utilize escape mechanisms to avoid immune recognition and destruction. This data implies that chondrosarcoma will benefit from strategies that enhance the immunogenicity of tumor antigens and/or counteract the escape mechanisms.

INTRODUCTION

Conventional chondrosarcoma is the secondmost common primary malignancy of bone (1). The clinical course of this disease including development ofmetastases is closely associated with histologic grade. Whereas low-grade conventional chondrosarcoma has a favorable prognosis, high-grade conventional chondrosarcoma, and in particular, dedifferentiated chondrosarcoma, has a poor outcome and a high tendency to metastasize (2, 3). Since conventional radiation and chemotherapy are ineffective, surgical resection is the standard of care for primary conventional and dedifferentiated chondrosarcoma. No effective systemic therapies for the treatment of metastatic disease are available (4, 5).

Major progress has been recently made in the development of immunotherapeutic strategies for the treatment of malignant diseases (6, 7). Impressive clinical responses have been convincingly documented in some of the treated patients with several types of cancer (8, 9). These clinical findings have stimulated interest in the development and application of immunotherapeutic strategies for the treatment of chondrosarcoma (10, 11).

Immunotherapy aims at influencing and/or enhancing a tumor antigen-specific immune response in a host with the expectation that it will eliminate cancer cells. Essentially all elements of the host's acquired and innate immune system participate in an effective tumor antigen-specific immune response. Preclinical and clinically-based studies have shown beneficial responses to be highly dependent on the induction and functional activity of cytotoxic CD8+ T lymphocytes, which recognize tumor antigen(s) expressed by tumor cells. These antigens are processed and presented on tumor cell surface in the context of HLA class I molecules. In turn, CD8+ T cells require the functional activities of CD4+ T helper cells, which interact with a range of immune cells, such as antigen presenting cells (APC) and macrophages that present tumor antigens in the context of HLA class II molecules (12). However, cancer cells also develop multiple escape mechanisms to avoid immune surveillance and abrogate potentially effective host tumor antigen-specific immune responses. Foremost are defects in antigen processing and presenting machinery, which include defects in HLA class I subunits, namely, HLA class I heavy chain and beta-2 microglobulin (β2m) and in HLA class II molecules (13–15). More recently, attention has been focused on immune checkpoint molecules, which normally help prevent inappropriate targeting of normal cells by the host's immune system (16). However, many cancer cells have co-opted these checkpoint inhibitors in order to prevent their destruction by CD8+ and CD4+ tumor infiltrating lymphocytes (TILs) (16). The immune checkpoint molecules, B7-H3 and PD-1/PD-L1, are currently being actively investigated in clinical studies and/or are currently used as targets of therapeutic strategies for the treatment of

many human cancer types (17–20). B7-H3 is an immune checkpoint molecule with limited expression in normal tissues (21) which has most commonly been associated with inhibition of cytotoxic CD8+ T lymphocyte activation (22) by tumor cells presenting tumor antigens on HLA class I antigen complexes. The other checkpoint molecule, PD-L1, is expressed on normal and cancer cells and appears to release inhibitory signals upon its interaction with its receptor PD-1 on T cells and thereby weakens their reactivity (17, 23).

Limited information is available about the role of immunological events in the pathogenesis and clinical course of chondrosarcoma. The lack of this information has a negative impact on the development of immunotherapies, which have been shown to be very effective in other types of previously recalcitrant cancers. The aim of the current study was to investigate the presence of immune cells and the expression of a selected set of immunologically relevant molecules in a tissue microarray consisting of conventional and dedifferentiated chondrosarcoma specimens and control tissues. The parameters chosen were 1) CD8+ and CD4+ TILs 2) HLA class I heavy chain and β2m, 3) HLA class II and 4) immune checkpoint B7-H3 and PD-1/PD-L1 molecules. The detection of TILs in the tumor microenvironment would be compatible with the possibility that a host's immune system recognizes and mounts an immune response against tumor antigens expressed on chondrosarcoma cells. Abnormalities in HLA expression and expression of checkpoint molecules would suggest potential escape mechanisms utilized by chondrosarcoma cells to avoid immune recognition and destruction.

PATIENTS, MATERIAL AND METHODS

Patient Characteristics and Tumor Specimens

This study represents a retrospective evaluation of tumor specimens obtained from a cohort of 76 patients with chondrosarcoma with a mean age of 56 years (range, 18-83); 51% were male. They were treated at Massachusetts General Hospital (MGH) during a 20-year period (1993 to 2013). The patients were included in the study if they had a minimum of 2 years of follow-up or until death and sufficient paraffin embedded tumor tissue was available for the construction of the tissue microarray. Patient information collected on each patient included: age, gender, margin status, tumor grade and presence of metastases. This study was approved by the Institutional Review Board at MGH (approval number 2013P001012). Table 1 summarizes the clinicopathologic characteristics of the patients and their tumors. The tumors included conventional and dedifferentiated chondrosarcoma as confirmed by 2 senior musculoskeletal pathologists (G.P. N. and V. D.) according to the WHO classification

system (24). Of the 76 patients selected for the study, 24 were diagnosed with a dedifferentiated chondrosarcoma.

Chondrosarcoma Tissue Microarray (TMA)

The TMA was constructed using 4-millimeter (mm) diameter cores extracted from representative regions of tumor blocks. In addition to the primary tumor specimens of 76 patients, 8 out of the 76 patients had both primary and corresponding metastatic tissue available for embedding in the TMA. Three of these metastases were from patients with conventional chondrosarcoma and 5 were from patients with dedifferentiated chondrosarcoma. In addition we included 8 randomly selected enchondroma in our TMA. Control tissues of human spleen, human cartilage, human liver, human lymph node, human melanoma metastasis, melanoma xenografts and mouse liver were also included in construction of the TMA. Four mm formalin-fixed, paraffin-embedded tissue sections from the TMA block were used as substrates in immunohistochemical staining. All of the prepared TMA sections contained the full complement of tumor tissue for analysis of each immune parameter being analyzed. The total number of tumor cores suitable for analysis varied due to confined amount of reliable interpretable tissue following specific experiments, as indicated by the number of samples utilized for each marker (in text, figures and tables). When we couldn't reliably interpret the staining we excluded the data of staining of the particular case from the analysis as indicated.

Monoclonal Antibodies (mAb)

The human CD8 (clone 4B12) and CD4 (EPR6844) specific mAb were purchased from DAKO (Carpinteria, CA, USA) and Abcam (Cambridge, MA, USA), respectively.

The mAb HC-A2, which recognizes β2m -free HLA-A (excluding -A24), -B7301, and -G heavy chains (25, 26); mAb HC-10, which recognizes β2m -free HLA-A3, -A10, -A28, -A29, -A30, A31, -A32, -A33, and all β2m -free -HLA-B (excluding -B5702, -B5804, and -B73) and -HLA-C heavy chains (25–27), β2m -specific mAb NAMB1 (28) and mAb LGII-612.14 which recognizes a monomorphic epitope expressed on the b chain of HLA DR, -DQ, and –DP antigens (29), were developed as described before. mAbs were purified from ascitic fluid by affinity chromatography on a Protein G column (GE Healthcare Life Sciences, Pittsburgh, PA). The purity and activity of mAb preparations were controlled by SDS-PAGE and by binding assays with the cognate antigen, respectively. The B7-H3 specific mAb 1027 was purchased from R&D System (Minneapolis, MN, USA) (30–32). The PD-L1-specific mAb clone 22C3 was developed by Merck Research Laboratories; and the PD-1 specific mAb clone NAT105, was purchased from Cell Marque (33).

Immunohistochemical Staining of Chondrosarcoma TMA

Staining with CD8- and CD4-specific mAb was performed according to the manufacturers' instructions. Results were calculated by counting the number of stained infiltrating cells within the tumor tissue in a 200x magnification. Lymphocytes were counted in a high-power field that was placed randomly in the tumor tissue. Depending on the amount of tumor tissue the field was placed a maximum of 4 times per tumor core. The total number of lymphocytes was counted and then summed up and was subsequently divided by the number of high-power fields that were counted in the multiple cores in each tumor. This resulted in an actual mean lymphocytic infiltration per highpower-field per tumor.

The immunohistochemical staining of TMA sections with HC-A2, HC-10, NAMB1, LGII-612.14 and B7-H3 mAb was performed as described previously (34). The percentage of stained tumor cells and staining intensity in each lesion were assessed by an investigator who had no knowledge of the patients' characteristics and clinical outcomes. Results were scored as positive, heterogeneous, or negative when the percentage of stained tumor cells in an entire lesion was greater than 75%, 75% to 25%, and less than 25%, respectively (35). The staining with the anti–PD-1 and anti–PD-L1 mAb was performed by Merck Research Laboratories as described (33). Heat-induced epitope retrieval (HIER) was performed for staining for PD-1 and PD-L1. Slides were immersed in FLEX High pH target retrieval solution for 20 minutes at 97°C (cat. K8012; DAKO). Slides were incubated in 3% hydrogen peroxide solution to block endogenous peroxidase in the tissues before incubating them for 60 minutes at room temperature with the primary antibody. Visualization of the antigen-antibody binding was performed by application of the FLEX+ polymer system (cat. K8012; DAKO) and by application of DAB chromagen (cat. K4368; DAKO). Counterstaining with hematoxylin was used on the stained slides.

Decalcified tissue was excluded from the analysis for PD-1 and PD-L1 since the results of staining decalcified tissue were not reproducible.

Statistical Analysis

Differences in contingency tables were investigated with Fisher's exact test and correlations were displayed with Spearman's rank correlation coefficients. Mann–Whitney U test was applied to investigate differences in two groups of continuous data. To analyze time to survival concerning overall survival and time to metastasis the log-rank test of equality across strata was applied and cox regression analysis was used for continuous variables. The statistical analysis was performed with the use of STATA 12 software, (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP.).

Table 1: Clinical and Pathological characteristics of the Analyzed Chondrosarcoma Tumors

Grade 1. (Sample # = 17)			**Grade 2. (Sample # = 30)**		
	Mean ± SD	**range**		**Mean ± SD**	**range**
Age, years (n=17)	49 ± 14	18-69	Age, years (#=30)	57 ± 16	33-83
Sex	**#**	**%**	**Sex**	**#**	**%**
male	6	35	male	15	50
female	11	65	female	15	50
	Mean ± SD	**range**		**Mean ± SD**	**range**
Tumor Size, cm3 (n=14)	137 ± 235	1.2-900	Tumor Size, cm3 (n=25)	705 ± 1253	1.8-5888
Anatomic Site	**#**	**%**	**Anatomic Site**	**#**	**%**
Spine	2	12	Spine	0	0
Scapula/clavicle	3	18	Scapula/clavicle	3	10
Sternum	1	5.9	Sternum	0	0
Rib(s)	0	0	Rib(s)	2	6.7
Humerus	1	5.9	Humerus	5	17
Pelvis	1	5.9	Pelvis	5	17
Sacrum	2	12	Sacrum	2	6.7
Femur	5	29	Femur	10	33
Tibia	0	0	Tibia	2	6.7
Margin, mm	**#**	**%**	**Margin**, mm	**#**	**%**
0	3	18	0	4	13
<1	3	18	<1	6	20
≥1	8	47	≥1	19	63
unknown	3	18	unknown	1	3.3
Extra-osseos extension	**#**	**%**	**Extra-osseous extension**	**#**	**%**
yes	9	53	yes	24	80
no	6	35	no	6	20
unknown	2	12	unknown	0	0
Metastasis at presentation	**#**	**%**	**Metastasis at presentation**	**#**	**%**
yes	0	0	yes	0	0
no	17	100	no	30	100

Metastasis	**#**	**%**	**Metastasis**	**#**	**%**
yes	0	0	yes	6	20
no	17	100	no	24	80

Local recurrence	**#**	**%**	**Local recurrence**	**#**	**%**
yes	2	12	yes	7	23
no	15	88	no	23	77

Grade 3. (Sample # = 5)			**Dedifferentiated (Sample # = 24)**		
	Mean ± SD	**range**		**Mean ± SD**	**range**
Age, years(#=5)	56 ± 13	39-73	Age, years (#=24)	59 ± 13	39-82
Sex	**#**	**%**	**Sex**	**#**	**%**
male	3	60	male	15	63
female	2	40	female	9	38

	Mean ± SD	**range**		**Mean ± SD**	**range**
Tumor Size, cm3 (n=5)	463 ± 499	36-1248	Tumor Size, cm3 (n=21)	591 ± 875	12-3680

Anatomic Site	**#**	**%**	**Anatomic Site**	**#**	**%**
Spine	1	20	Spine	1	4.2
Scapula/clavicle	0	0	Scapula/clavicle	2	8.3
Sternum	0	0	Sternum	0	0
Rib(s)	0	0	Rib(s)	1	4.2
Humerus	0	0	Humerus	2	8.3
Pelvis	3	60	Pelvis	5	21
Sacrum	0	0	Sacrum	0	0
Femur	1	20	Femur	12	50
Tibia	0	0	Tibia	1	4.2

Margin, mm	**#**	**%**	**Margin**, mm	**#**	**%**
0	2	40	0	8	33
<1	2	40	<1	5	21
≥1	1	20	≥1	10	42
unknown	0	0	unknown	1	4.2

Extra-osseous extension	**#**	**%**	**Extra-osseous extension**	**#**	**%**
yes	5	100	yes	22	92
no	0	0	no	1	4.2
unknown	0	0	unknown	1	4.2

Metastasis at presentation	#	%	Metastasis at presentation	#	%
yes	0	0	yes	7	29
no	5	100	no	17	71

Metastasis	#	%	Metastasis	#	%
yes	2	40	yes	22	92
no	3	60	no	2	8.3

Local recurrence	#	%	Local recurrence	#	%
yes	1	20	yes	7	29
no	4	80	no	17	71

RESULTS

Higher Level of TILs in High-Grade Than in Low-Grade Conventional Chondrosarcoma Tumors

To investigate whether patients with chondrosarcoma developed an immune response to the tumor antigens expressed by their tumors, the chondrosarcoma TMA was analyzed for the presence of CD4+ and CD8+ TILs. CD4+ T cells were present in 21% of the 61 chondrosarcoma tumors analyzed and CD8+ T cells were present in 44% of the 62 chondrosarcoma tumors analyzed (conventional and dedifferentiated combined). The number of CD4+ T cells detected ranged between 0.0 and 7.9 lymphocytes per highpower field (mean n=0.16 ± 1.0, n=61), whereas that of CD8+ T cells ranged between 0.0 and 52 lymphocytes per high-power field (mean n=5.0 ± 11, n=62) in chondrosarcoma (conventional and dedifferentiated combined). Representative staining patterns of chondrosarcoma tumors with CD4+ and CD8+ mAb are shown in Figure 1. No correlation between levels of CD4+ and CD8+ TILs was detected (p=0.57). A comparison of the TILs within conventional chondrosarcoma showed that grade 3 tumors had higher CD8+ TILs than grade 1 or 2 tumors: mean 3.4 ± 6.1 (range, 0-14, n=5) lymphocytes per high-power field versus 0.26 ± 1.3 (range, 0-7.6, n=37) lymphocytes per high-power field, (p=0.014).

Higher Level of TILs in Dedifferentiated Chondrosarcoma Than in Conventional Chondrosarcoma Tumors

The level of CD8+ TILs in conventional chondrosarcoma tumors was lower than that in the dedifferentiated tumors (21% (9 out of 42 conventional chondrosarcoma analyzed) vs. 90% (18 out of 20 dedifferentiated chondrosarcoma analyzed), P<0.0001). There was no statistical difference in CD4+ TILs between conventional and dedifferentiated chondrosarcoma (p=0.90). In a univariable analysis, presence of CD8+ TILs in

chondrosarcoma (conventional and dedifferentiated combined) was associated with higher risk of mortality (HR=1.1, 95% CI [1.0-1.1], p<0.001). However, in a multivariable analysis controlling for dedifferentiated versus conventional chondrosarcoma, there was no statistical association of CD8+ TIL number with survival (HR=1.0, 95% CI [0.98-1.0], p=0.59).

Higher HLA Class I Expression in High-Grade Than in Low-Grade Conventional Chondrosarcoma Tumors

In conventional chondrosarcoma, HLA-A heavy chain expression was scored positive in 8% (4 out of 50 tumors analyzed), heterogeneous in 42% (21 out of 50 tumors analyzed) and negative in 50% (25 out of 50 tumors analyzed). HLA-B, -C heavy chain expression was scored positive in 23% (12 out of 52 tumors analyzed), heterogeneous in 40% (21 out of 52 tumors analyzed) and negative in 37% (19 out of 52 tumors analyzed) of the tumors (Table 2).

HLA-A heavy chain expression was significantly higher in grade 2 and grade 3 conventional chondrosarcoma (high-grade) than in grade 1 chondrosarcoma (low-grade): 39% ± 23 (mean expression of 34 grade 2 and grade 3 tumors analyzed, range 10-83%) versus 16% ± 13 (mean expression of 16 grade 1 tumors analyzed, range 10-60%), p=0.0003. Also HLA-B, -C heavy chain expression was significantly higher on average in higher-grade conventional chondrosarcoma than in grade 1 chondrosarcoma: 49% ± 25 (mean expression of 35 grade 2 and grade 3 tumors analyzed, range 10-85%) versus 27% ± 21 (mean expression of 17 grade 1 tumors analyzed, range 10-75%), p=0.0053.

Higher HLA Class I Expression in Dedifferentiated Than in Conventional Chondrosarcoma Tumors

In dedifferentiated chondrosarcoma, HLA-A heavy chain expression was scored positive in 85% (17 out of 20 tumors analyzed) and heterogeneous in 15% (3 out of 20 tumors analyzed) of the tumors, HLA-B, -C heavy chain expression was scored positive in 100% of the tumors (20 out of 20 tumors analyzed).

Overall, the mean percentage of HLA-A heavy chain expression was significantly higher in dedifferentiated than in conventional chondrosarcoma: 83% ± 10 (mean expression of the 20 dedifferentiated chondrosarcoma analyzed, range 55-90%) versus 31% ± 23 (mean expression of the 50 conventional chondrosarcoma analyzed, range 10-83%), p<0.0001. Similarly, the mean percentage of HLA-B, -C heavy chain expression was significantly higher in dedifferentiated than in conventional chondrosarcoma: 90% ± 1.5 (mean expression of the 20 dedifferentiated chondrosarcoma analyzed, range 85-90%) versus 42% ± 26 (mean expression of the 52 conventional chondrosarcoma analyzed, range 10-85%), p<0.0001. Representative staining patterns of expression of HLA-A heavy chain with HC-A2 mAb and HLA-B, -C heavy chain with HC-10 mAb are shown in Figure 2.

Table 2: HLA Class I subunit and HLA Class II antigen expression in Benign and Malignant Cartilage Tumors

		Negative				Heterogeneous				Positive			
Enchondroma	HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)
	A Heavy Chain	4	10	0	10-10	4	44	8.7	32-50	0	.	.	.
	B, C Heavy Chain	2	10	0	10-10	3	51	15	37-67	2	75	0	75-75
	B2m	4	10	0	10-10	2	44	1.2	43-45	0	.	.	.
	Class II	6	13	6.1	10-25	0	.	.	.	0	.	.	.
Grade 1. chondrosarcoma	HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)
	A Heavy Chain	14	11	2.7	10-20	2	48	18	35-60	0	.	.	.
	B, C Heavy Chain	11	15	5.8	10-25	4	38	9.9	30-50	2	75	0	75-75
	B2m	11	11	2.2	10-17	5	41	9.4	30-52	0	.	.	.
	Class II	15	10	0	10-10	0	.	.	.	0	.	.	.
Grade 2. chondrosarcoma	HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)
	A Heavy Chain	10	14	5.6	10-25	15	42	13	27-68	4	79	4.3	75-83
	B, C Heavy Chain	7	13	5.0	10-23	15	46	11	30-63	8	77	4.1	75-85
	B2m	14	16	5.3	10-25	11	46	10	33-67	5	81	5.0	75-87
	Class II	26	13	3.9	10-25	2	41	20	27-55	0	.	.	.
Grade 3. chondrosarcoma	HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)
	A Heavy Chain	1	10	.	10-10	4	54	12	36-62	0	.	.	.
	B, C Heavy Chain	1	15	.	15-15	2	68	7.1	63-73	2	78	3.5	75-80
	B2m	1	10	.	10-10	4	45	9.8	35-57	0	.	.	.
	Class II	5	17	5.6	10-25	0	.	.	.	0	.	.	.

		Negative				Heterogeneous				Positive			
Dedifferentiated chondrosarcoma	HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)
	A Heavy Chain	0	.	.	.	3	64	9.2	55-73	17	86	5.9	75-90
	B, C Heavy Chain	0	.	.	.	0	.	.	.	20	90	1.5	85-90
	B2m	2	10	0	10-10	10	51	8.8	37-60	7	86	3.0	83-90
	Class II	0	.	.	.	10	52	12	33-70	10	83	6.4	75-90

HLA-A heavy chain expression showed a positive and strong correlation with that of HLA-B, -C heavy chain expression (Spearman's coefficient of 0.88 (n=77), $p<0.0001$). The presence of CD8+ TILs was correlated with HLA-A heavy chain (Spearman's coefficient of 0.61 (n=68), $p<0.0001$) and HLA-B, -C heavy chain (Spearman's coefficient of 0.71 (n=68), $p<0.0001$).

Table 3a: Higher B7-H3 expression in dedifferentiated than in conventional chondrosarcoma

	Sample #	Negative Staining	Heterogeneous Staining	Positive Staining
Grade 1	15	11	3	1
Grade 2	28	3	11	14
Grade 3	5	1	1	3
Dedifferentiated	24	1	2	21

Table 3b: Higher PD-L1 in dedifferentiated than in conventional chondrosarcoma.

	Sample #	No expression	Positive expression
Grade 1	6	6	0
Grade 2	19	19	0
Grade 3	4	4	0
Dedifferentiated	15	10	5

Higher β2m Expression in High-Grade Than in Low-Grade Conventional Chondrosarcoma Tumors

In conventional chondrosarcoma β2m expression was scored positive in 10% (5 out of 51 tumors analyzed), heterogeneous in 39% (20 out of 51 tumors analyzed) and negative in 51% (26 out of 51 tumors analyzed) of the tumors analyzed. In addition, the mean percentage of β2m expression was significantly lower in grade 1 tumors than in grade 2 and grade 3 chondrosarcoma: 21% ± 15 (mean expression of 16 grade 1 tumors analyzed, range 10-52%) versus 38% ± 24 (mean expression of 35 grade 2 and grade 3 tumors analyzed, range 10-87%), p=0.0048. Representative staining patterns with β2m-specific NAMB1 mAb are shown in Figure 2.

Higher β2m Expression in Dedifferentiated Chondrosarcoma Than in Conventional Chondrosarcoma Tumors

In dedifferentiated chondrosarcoma β2m expression was scored positive in 37% (7 out of 19 tumors analyzed), heterogeneous in 53% (10 out of 19 tumors analyzed) and negative in 11% (2 out of 19 tumors analyzed) of the tumors (Table 2). This pattern was similar to that found for HLA class I heavy chains in these 2 types of chondrosarcoma. The mean percentage of β2m expression was significantly lower in conventional chondrosarcoma than in dedifferentiated tumors: 32% ± 23 (mean expression of the 51 conventional chondrosarcoma analyzed, range 10-87%) versus 59% ± 25 (mean expression of the 19 dedifferentiated chondrosarcoma, range 10-90%), p=0.0002. Analysis of conventional chondrosarcoma showed that β2m expression is positively and significantly correlated with that of HLA-A heavy chain (Spearman's coefficient of 0.72, $p<0.0001$) and of HLA-B, -C heavy chain (Spearman's coefficient of 0.79, $p<0.0001$).

Higher HLA Class II Expression in High-Grade Than in Low-Grade Conventional Chondrosarcoma Tumors

In conventional chondrosarcoma, HLA class II expression was scored heterogeneous in 4% (2 out of 48 tumors analyzed) and negative in 96% (46 out of 48 tumors analyzed). HLA class II expression was lower in grade 1 tumors than in grade 2 and 3 chondrosarcoma: 10% ± 0 (mean expression of 15 grade 1 tumors analyzed, range 10-10%) versus 15% ± 8.7 (mean expression of 33 grade 2 and grade 3 tumors analyzed, range 10-55%), p=0.0005.

Higher HLA Class II Expression in Dedifferentiated Chondrosarcoma Than in Conventional Chondrosarcoma Tumors

In differentiated chondrosarcoma, HLA class II expression was scored positive in 50% (10 out of 20 tumors analyzed) and heterogeneous in 50% (10 out of 20 tumors analyzed) (Table 2).

Overall, HLA class II expression was significantly higher in dedifferentiated chondrosarcoma than in conventional chondrosarcoma: 67% ± 19 (mean expression of 20 dedifferentiated chondrosarcoma analyzed, range 33-90%) versus 14% ± 7.5 (mean expression of 48 conventional chondrosarcoma analyzed, range 10-55%), $p<0.0001$.

Higher HLA Class I, β2m and HLA Class II Expression in the Dedifferentiated Component Than in the Conventional Component Within Dedifferentiated Chondrosarcoma

In dedifferentiated chondrosarcoma, the HLA-A heavy chain expression of the conventional (low-grade) component was scored positive in 13% (2 out of 15 tumors analyzed), heterogeneous in 20% (3 out of 15 tumor analyzed) en negative in 67% (10 out of 15 tumors analyzed).

The HLA-B, -C heavy chain expression of the conventional component was scored positive in 17% (2 out of 12 tumors analyzed), heterogeneous in 17% (2 out of 12 tumors analyzed) and negative in 67% (8 out of 12 tumors analyzed).

The β2m expression of the conventional component within the dedifferentiated chondrosarcoma was scored positive in 7.7% (1 out of 13 tumors analyzed), heterogeneous in 23% (3 out of 13 tumors analyzed) and negative in 69% (9 out of 13 tumors analyzed).

The HLA class II expression of the conventional component was scored negative in 100% (13 out of 13 tumors analyzed).

Matched analysis of the expression of HLA class I, β2m and HLA class II between the conventional component versus the dedifferentiated component showed higher HLA-A ($p=0.0030$), HLA-BC ($p=0.014$), β2m ($p=0.050$) and HLA class II ($p=0.0076$) in the dedifferentiated component versus the conventional component in the same tumor.

Table 4: TIL, HLA Class I subunit and HLA Class II antigen expression in metastasis and in primary tumors

a. combined dedifferentiated and conventional chondrosarcoma combined

Primary					**Metastasis**					
	#	Mean (cell #)	SD (%)	Range (%)	#	Mean (cell #)	SD (%)	Range (%)	**Observation**	**P-value**
CD4+	61	0.16	1.0	0-7.9	7	0.080	0.11	0-0.25	Lower	0.090
CD8+	62	5.0	11	0-52	6	2.7	4.4	0-11	Lower	0.16
HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)		
A Heavy Chain	70	46	31	10-90	6	66	27	20-90	Higher	0.59
B, C Heavy Chain	72	55	31	10-90	7	83	7.6	75-90	Higher	0.17
B2m	70	40	26	10-90	7	62	24	30-90	Higher	0.22
Class II	68	29	27	10-90	7	53	33	10-83	Higher	0.17

b. conventional chondrosarcoma

Primary					**Metastasis**					
	#	Mean (cell #)	SD (%)	Range (%)	#	Mean (cell #)	SD (%)	Range (%)	**Observation**	**P-value**
CD4+	39	0.030	0.059	0-0.20	3	0.10	0.091	0-0.17	Higher	0.17
CD8+	42	0.63	2.5	0-14	3	0	NA	0-0	Lower	NA
HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)		
A Heavy Chain	50	31	23	10-83	3	49	30	20-80	Higher	0.29
B, C Heavy Chain	52	42	26	10-85	3	78	5.8	75-85	Higher	0.11
B2m	51	32	23	10-87	3	57	25	30-80	Higher	0.29
Class II	48	14	7.5	10-55	3	19	16	10-38	Higher	0.78

c. dedifferentiated chondrosarcoma

Primary					**Metastasis**					
	#	Mean (cell #)	SD (%)	Range (%)	#	Mean (cell #)	SD (%)	Range (%)	**Observation**	**P-value**
CD4+	22	0.39	1.7	0-7.9	4	0.063	0.13	0-0.25	Lower	0.32
CD8+	20	14	15	0-52	3	5.4	5.1	0.5-11	Lower	0.18
HLA	#	Mean (%)	SD (%)	Range (%)	#	Mean (%)	SD (%)	Range (%)		
A Heavy Chain	20	83	10	55-90	3	83	7.6	75-90	Equal	0.65
B, C Heavy Chain	20	90	1.5	85-90	4	86	7.5	75-90	Lower	0.32
B2m	19	59	25	10-90	4	67	26	30-90	Higher	0.65
Class II	20	67	19	33-90	4	78	4.1	75-83	Higher	0.18

Higher B7-H3 Expression in Dedifferentiated Chondrosarcoma Than in Conventional Chondrosarcoma Tumors

In conventional chondrosarcoma, B7-H3 expression was scored positive in 38% (18 out of 48 tumors analysed), heterogeneous in 31% (15 out of 48 tumors analysed) and negative in 31% (15 out of 48 tumors analysed). In dedifferentiated chondrosarcoma, B7-H3 expression was scored positive in 88% (21 out of 24 tumors analysed), heterogeneous in 8% (2 out of 24 tumors analysed) and negative in 4% (1 out of 24 tumors analysed), (Table 3). In conventional chondrosarcoma, there were significant differences among 3 B7-H3 expression levels (negative, heterogeneous and positive) and tumor grade ($p<0.0001$) Higher-grade tumors showed higher B7-H3 expression levels.

Higher PD-L1 in Dedifferentiated Chondrosarcoma Tumors Than in Conventional Chondrosarcoma Tumors

Eight out of the 15 (53%) patients analyzed with dedifferentiated tumors expressed

the immune checkpoint PD-1 antigen on their TILs (excluding the 9 patients with decalcified tissue) (Table 3). The mean expression of these 8 patients was 2.8 ± 1.5 (range, 1-5) on a scale to 5. PD-L1 was expressed in 5 out of the 15 dedifferentiated tumors (33%) with a mean expression of 4.8 ± 0.45 (range, 4-5) on a scale to 5 (Table 3). PD-1 expression was correlated with that of PD-L1 expression in the dedifferentiated chondrosarcoma (Spearman's rho = 0.80, $p<0.0003$) (Table 3).

Representative staining patterns of PD-1 and PD-L1 are shown in Figure 2. No staining by the PD-L1-specific mAb was detected in any of the analyzed conventional chondrosarcoma (n=29, excluding decalcified tissue). We did not stain the conventional chondrosarcoma for PD-1 since none of the conventional chondrosarcoma expressed any PD-L1 (Table 3).

Positive staining of PD-L1 was associated with a significantly shorter time to metastasis ($p=0.019$) (excluding the patients with metastasized disease at the time of presentation). The mean time to metastasis was 1.6 months, 95% CI [0.032-3.1], for the tumors stained by PD-L1 specific mAb tumors (n=2) but 20 months, 95% CI [1.8-95] for the tumors with no detectable PD-L1 staining (n=12).

Figure 1. Representative immunohistochemical staining patterns of dedifferentiated chondrosarcoma tumors with lymphocyte and checkpoint-specific monoclonal antibodies.

A; $CD4^+$ TILs (200x magnification). B; $CD8^+$ TILs (200x magnification). C; PD-L1 positive cells (100x magnification). D; PD-L1 positive cells (200x magnification). E; PD-L1 positive cells (200x magnification). F; PD-L1 positive cells (200x magnification). G; PD-1 positive lymphocytes (200x magnification). H; PD-1 positive lymphocytes (200x magnification).

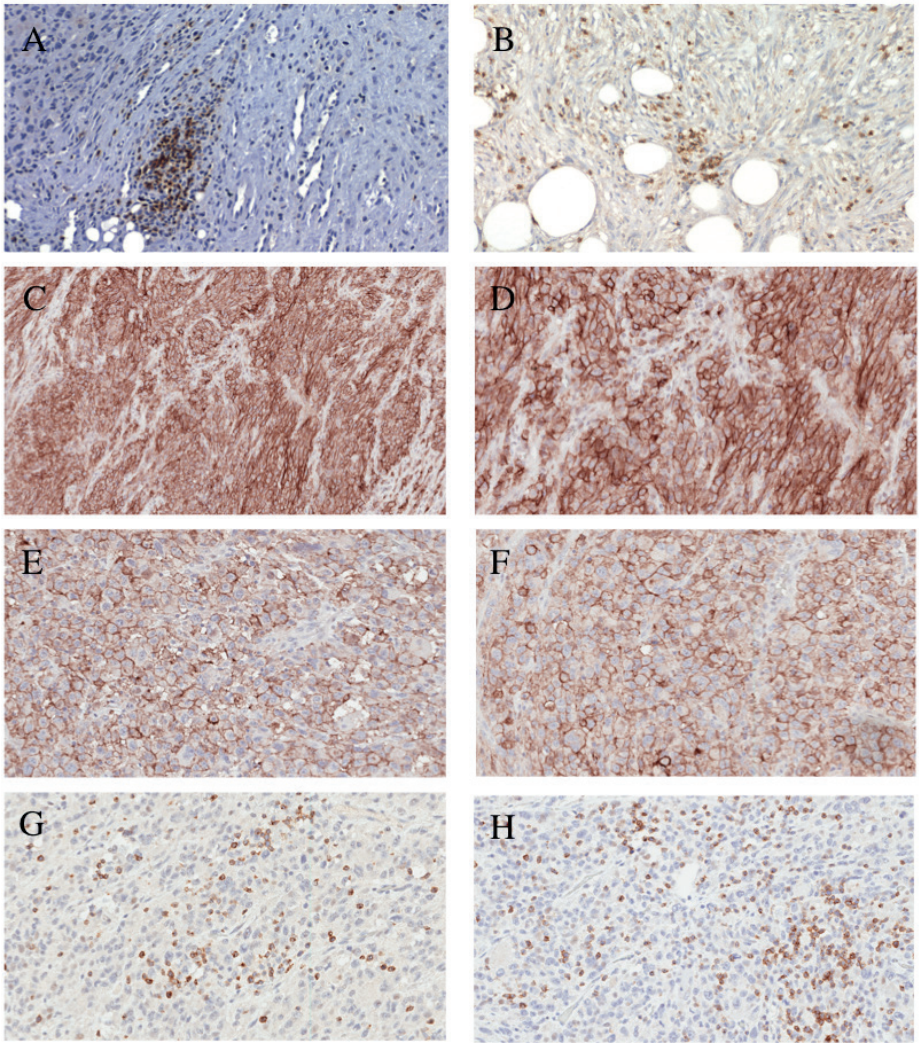
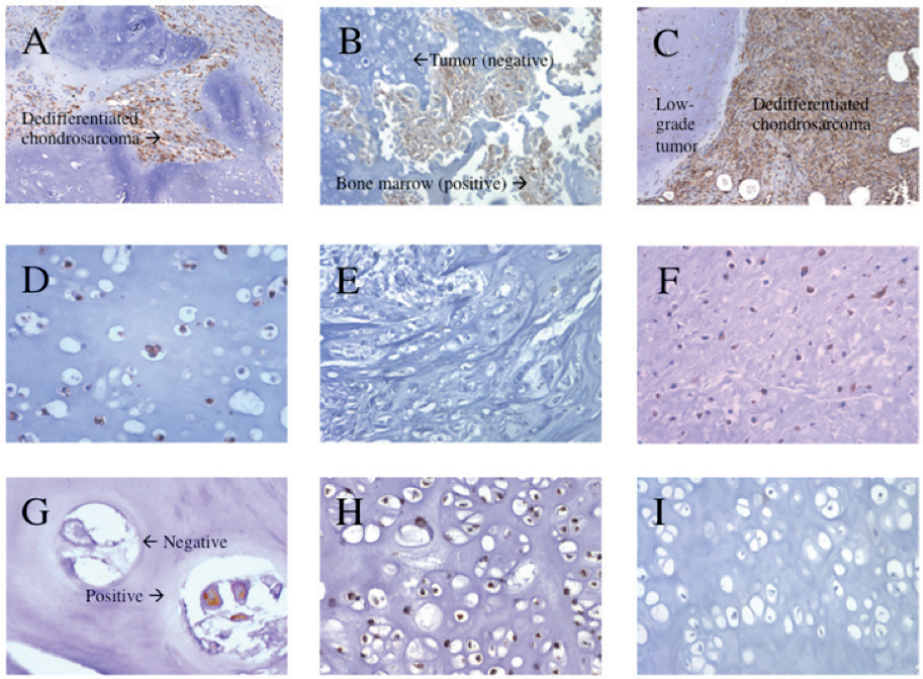


Figure 2. Representative immonohistochemical staining patterns of primary chondrosarcoma tumors with HLA class I subunit specific monoclonal antibodies.

A; HLA-A positive stain in dedifferentiated chondrosarcoma (100x magnification). B; HLA-A negative stain in grade 1 chondrosarcoma with positive bone marrow (200x magnification). C; HLA-B, -C positive stain in dedifferentiated chondrosarcoma (100x magnification). D; HLA-B, -C positive stain in grade 2 chondrosarcoma (400x magnification). E; HLA-B, -C negative stain in grade 3 chondrosarcoma (200x magnification). F; HLA-B, -C positive stain in grade 3 chondrosarcoma (200x magnification). G; β2m both a positive and negative stain in grade 1 chondrosarcoma (400x magnification). H; β2m positive stain in grade 2 chondrosarcoma (200x magnification). I; β2m negative stain in grade 2 chondrosarcoma (200x magnification).



Comparison of the Immunohistochemical Staining Profile of Primary and Autologous Metastatic Chondrosarcoma Lesions in Conventional and Dedifferentiated Chondrosarcoma

The expression of HLA class I antigens, HLA class II antigens and checkpoint molecules in 8 primary and autologous metastatic lesions was compared. Of the 8 primary lesions, 5 were dedifferentiated, 2 were grade 2 and 1 was grade 3 conventional chondrosarcoma. HLA class I and HLA class II expression in the autologous metastases appeared to be higher than in the primary tumors; however, this difference was not statistically significant and was not associated with higher TILs in metastases.

No difference in mean PD-1 and PD-L1 expression was detected between primary and autologous metastatic lesions.

Due to the low number of cases, a meaningful statistical analysis was not possible, but a descriptive table of the results of the analysis of the 8 cases stratified according to tumor grade is part of Table 4.

DISCUSSION

Our study tested whether patients with chondrosarcoma develop a T cell immune response against their own tumor and we have analysed the expression of immunologically relevant molecules on chondrosarcoma cells. The latter include HLA class I subunits, HLA class II antigens and checkpoints B7-H3 and PD-L1. The resulting information contributes to our understanding of the role of immune surveillance. We showed that low-grade (grade 1) conventional chondrosarcoma are less immunogenic as indicated by limited TILs and lower HLA class I and HLA class II expression compared to high-grade conventional chondrosarcoma (grade 2 and 3). In addition, dedifferentiated chondrosarcoma are more immunogenic compared to conventional chondrosarcoma as indicated by more TILs and higher HLA class I and HLA class II expression.

In this study, HLA class I antigen expression was decreased in 77-92% of the conventional chondrosarcoma, but in only 0-15% of the dedifferentiated chondrosarcoma. As observed in most, if not all the other types of solid cancer analysed (13, 36) defects in HLA class I antigen expression have been found in chondrosarcoma. In chondrosarcoma, the frequency of defects is significantly lower in subtypes with an aggressive phenotype and poor clinical course than in those with a benign phenotype and with a more favourable clinical course. This pattern is at variance with what has been found in most other types of solid cancer. In the latter defective HLA class I expression is in general associated with poor clinical course of the disease (13).

CD8+ TILs were present in 90% of dedifferentiated chondrosarcoma and 21% of conventional chondrosarcoma with high-grade conventional tumors having more CD8+ TILs than low-grade chondrosarcoma. It is intriguing that dedifferentiated chondrosarcoma carry an isocitrate dehydrogenase-1 (IDH1) and isocitrate dehydrogenase-2 (IDH2) mutation (37) with higher frequency than conventional chondrosarcoma since in glioma the neoantigens derived from IDH-mutation have been shown to induce a cellular immune response (38–42). Its inability to control tumor growth in chondrosarcoma may reflect the negative impact of the suppressive

tumor microenvironment, especially since in colorectal cancer an association has been found between B7-H3 and IDH1 expression level (43).

Several mechanisms can be envisioned for the association between defective HLA class I antigen expression and favourable course of the disease we have unexpectedly found in chondrosarcoma. One possibility is that immunosurveillance does not play a role in the pathogenesis and clinical course of the disease in chondrosarcoma. However this possibility is unlikely, since the lymphocyte infiltration we have found in tumors indicates that hosts recognize and develop a T cell mediated immune response to tumor antigens expressed by their own tumors. An alternative possibility is that in chondrosarcoma NK cells and not T cells play the major role in the elimination of malignant cells. If so, this mechanism would not be unique of chondrosarcoma, since it has already been described in other cancers (44, 45).

In this case high HLA class I antigen expression would represent a defensive mechanism for malignant cells, since it would very effectively inhibit the ability of NK cells to eliminate malignant cells. One last possibility that we favour is that patients mount an immune response to the tumor antigens expressed by their own tumors, however this response has no antitumor activity because it is inhibited by an immunosuppressive microenvironment. Our study demonstrated that the immune checkpoint molecule B7-H3 is expressed in 96% of dedifferentiated tumours and in 69% of conventional chondrosarcoma. Interestingly, B7-H3 expression was associated with high-grade versus lower grade conventional chondrosarcoma similar to our findings with HLA class I antigen expression. B7-H3 has been shown to inhibit the antitumor activity of T cells in many types of solid cancer has been associated with immune suppression and worse prognosis in multiple cancers, including glioblastoma, lung, renal cell carcinoma, and pancreatic ductal adenocarcinoma cancer (31, 46–48). B7-H3 has been shown to exert an inhibitory immune effect by preventing activation of CD8+ T lymphocytes (49). With a preferential expression on tumor cells and limited expression on normal tissue, B7-H3 is an attractive target for cancer immunotherapy (21, 50).

Another interesting result of our study is the association of PD-L1 expression on chondrosarcoma cells in primary tumors with shorter time to metastatic spreading. Caution has to be exercised in interpreting this finding because the number of tumor samples analysed is small, however in other cancers PD-L1 expression has been associated with metastatic disease (51–53).

Therefore, this association needs to be independently confirmed by analysing a large number of additional samples. If this association is corroborated by additional results and reflects a cause effect relationship, then one potential mechanism for the metastatic spreading is the escape of tumor cells from immunosurveillance because

of T cell exhaustion caused by the inhibitory signals triggered by the interactions between PD-1 expressed on T cells and PD-L1 expressed on chondrosarcoma cells and macrophages in the tumor microenvironment.

In our study, PD-L1 was expressed by 33% (5/15) of the dedifferentiated chondrosarcoma. In conventional chondrosarcoma there was no PD-L1 expression independently of tumor grade. Our findings are consistent with the information in the literature (54).

One might ask whether the results we have described have any clinical relevance especially in the area of therapy since this is an unmet need in chondrosarcoma. Our results can contribute to the rational design of immunotherapeutic strategies for the treatment of chondrosarcoma, especially since immunosurveillance appears to play a role in its clinical course. Therapies targeting B7-H3 and PD-1/PD-L1 axis may have a beneficial effect on this malignancy, since they may counteract the immunosuppression induced by B7-H3 and the PD-1 axis and may inhibit the metastatic potential of chondrosarcoma cells. This possibility is supported by two recent studies which have reported clinical responses in two patients with chondrosarcoma following treatment with nivolumab (55) or pembrolizumab (56).

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4

Chapter 4

Chondroitin sulfate proteoglycan 4 expression in chondrosarcoma: A potential target for antibody-based immunotherapy

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ABSTRACT

Purpose

Chondrosarcoma is a common primary bone malignancy whose phenotype increases with its histologic grade. They are relatively resistant to chemotherapy and radiation therapy limiting curative options for disseminated disease. Chondroitin sulfate proteoglycan 4 (CSPG4) is a cell surface proteoglycan that is highly expressed across various human cancers, including chondrosarcoma, and has restricted distribution in healthy tissues, making it an attractive target for the antibody-based therapy. CSPG4 specific chimeric antigen receptor (CAR) T cell therapies have been shown to be effective in treating other cancers such as melanoma and triple negative breast cancer. The goal of this study was to assess the prevalence of CSPG4 in human chondrosarcoma and to assess the efficacy of CSPG4 specific CAR T cells in lysing chondrosarcoma cells in vitro.

Patients and Methods

Using immunohistochemistry (IHC), we stained a tissue microarray containing primary conventional and dedifferentiated chondrosarcoma from 76 patients with CSPG4 specific monoclonal antibodies (mAbs). In addition, we incubated 2 chondrosarcoma cell lines with CSPG4-targeting CAR T cells and subsequently evaluated cell survival.

Results and Conclusion

Our results showed medium to high expression of CSPG4 in 29 of 41 (71%) conventional chondrosarcoma tumors and in 3 of 20 (15%) dedifferentiated chondrosarcoma tumors. CSPG4 expression showed a positive association with time to metastasis and survival in both subtypes. CSPG4 CAR T treated cell lines showed a lysis of respectively >80% and 70% demonstrating CSPG4-targeted CAR T cells effective in killing CSPG4-positive chondrosarcoma tumors.

INTRODUCTION

Chondrosarcoma is the second most common primary malignant cancer of the bone in which the tumor behavior and the histologic grade are closely associated (1, 2). Lowgrade chondrosarcoma are slow growing, may be locally aggressive, but carry a low risk of metastasizing. High-grade chondrosarcoma and dedifferentiated chondrosarcoma are aggressive and carry a high risk of early metastasis and recurrence (3, 4). The standard of care for chondrosarcoma remains complete surgical excision. There exists a need for effective systemic therapies in the case of locally recurrent disease, metastasic disease and when surgical resection leads to unacceptable morbidity to the patient. Chemotherapy and radiotherapy have not been effective in salvaging these cases and their use is generally limited to palliative settings (5, 6).

In previous studies, our group and others have shown that HLA I expression is reduced in conventional chondrosarcoma, suggesting that immunotherapeutic strategies targeting machinery upstream of tumor antigen cross-presentation will be ineffective in the absence of induced HLA I stimulation (7–9). This contrasts with dedifferentiated chondrosarcoma where limited defects in HLA I expression were observed and therefore potentially benefit from checkpoint blockade inhibition, a treatment approach that utilizes functional HLA I antigen expression (8, 10).

Here, we explore chimeric antigen receptor (CAR) T cell therapy, a tumor killing mechanism that is independent of HLAI antigen expression or functionality. CAR T cells express synthetic receptors that recognize and lyse cells expressing a specific target antigen. CAR binding to target antigens expressed on the cell surface is independent from the MHC receptors, yielding potent T cell activation and anti-tumor responses (11, 12). CAR T immunotherapy has been a remarkable success in leukemias with limited success in solid tumors (13–16), however, based on its MHC-independent activation of T cells, CAR T therapy represents a potential therapy in chondrosarcoma.

In this study, we focus on chondroitin sulfate proteoglycan 4 (CSPG4) as a potential immunotherapeutic target in chondrosarcoma based on the discovery that it is highly expressed in chemically-induced rat chondrosarcoma, and originally identified as rat chondroitin sulfate proteoglycan nerveglial 2 (NG2), later to be shown to share 100% homology with human CSPG4 (17–19). Earlier we have described the CSPG4 expression in chondrosarcoma and chordoma comparing the genetic profile of chondrosarcoma with other cancers (20). CSPG4 is a cell surface proteoglycan that is highly expressed across various types of human cancers with restricted expression in healthy tissues, making it an attractive target for antibody-based cancer immunotherapy. Importantly, CSPG4 specific monoclonal antibodies and adoptive

cell transfer therapies targeting CSPG4 have been effective in pre-clinical models by inhibition tumor growth and proliferation, making it a promising target for clinical application (18, 21–24). The clinical relevance of CSPG4 has been demonstrated in recent studies showing that this antigen is an independent risk factor for decreased survival in epithelial ovarian cancer (25).

The immunotherapeutic significance of CSPG4 in chondrosarcoma has not yet been explored and therefore we evaluated its efficacy as a potential target in chondrosarcoma immunotherapy by assessing the expression of CSPG4 in a large series of surgically removed conventional and dedifferentiated chondrosarcoma. We also investigated the association of CSPG4 expression with the overall survival and the development of metastases. In addition we investigated the effectiveness of CSPG4-specific CAR T cell therapy in eliminating chondrosarcoma cells in 2 chondrosarcoma cell lines.

MATERIALS AND METHODS

Clinical chondrosarcoma samples

We utilized the Orthopaedic Oncology registry at the Massachusetts General Hospital, Boston, Massachusetts to identify chondrosarcoma cases treated surgically from 1993 to 2013. We selected patient samples with a conventional or dedifferentiated chondrosarcoma in a non-cranial location tumor who had the primary tumor surgically removed at our hospital (IRB Approval #2013P001012).

Inclusion criteria

A two-year minimum clinical follow-up time or death was set for inclusion. Based on this criterion, we selected 82 subjects for further studies. Six of the 82 patient samples were excluded because of insufficient amount of paraffin embedded tumor tissue for the study. The sample size for our study was 76; 52 cases of diagnosed conventional chondrosarcoma ranging from grade 1-3 and 24 cases of dedifferentiated chondrosarcoma. The average age for the 76 patients in the cohort was 56 ± 14 years (range, 18-83) and 53% of these patients were male. The mean age for the grade 1, grade 2 and grade 3 patients were respectively 49 ± 14 years (range, 18-69), 57 ± 16 years (range, 33-83) and 56 ± 13 years (range, 39-73). The average age of patients with a dedifferentiated chondrosarcoma was 59 ± 13 years (range, 39-82) (Table 1).

Tissue microarray

Tissue blocks matched with hematoxylin and eosin-stained slides were obtained from the Department of Pathology, reviewed by 2 investigators (SN, JS) and independently confirmed by a senior musculoskeletal pathologist for adequate tissue. Areas to be

sampled were circled with a fine tipped felt pen. Multiple circles were made per sample to account for tissue heterogeneity. Circled areas on the slide were compared to the corresponding paraffin block to identify areas to be included in the tissue microarray (TMA) prior to assembly where 4 mm cylinders of representative regions were excised from each block. Eight primary-met pairs were included in the TMA representing 3 cases of conventional chondrosarcoma and 5 cases of dedifferentiated chondrosarcoma.

Eight enchondroma samples as well as clinical cartilage, spleen, liver and lymph node tissue were included as negative controls. Clinical melanoma metastasis sample, murine melanoma xenograft and liver samples were included as positive controls. Control samples were included to confirm staining specificity of monoclonal antibody (mAb) D2.8.5-C4B8. Tissue samples were placed randomly and individually arranged into a total of 6 paraffin blocks.

CSPG4 monoclonal antibody

The CSPG4-specific mAb D2.8.5-C4B8, a mouse IgG1, is secreted by a hybridoma generated from a BALB/c mouse immunized with a peptide corresponding to the amino acid sequence. The mAb was purified from ascitic fluid by affinity chromatography on a Protein G column (GE Healthcare Life Sciences, Pittsburgh, PA). The mAb preparation was controlled for purity and activity by SDS-PAGE and by binding assays with the cognate antigen. The mAb D2.8.5-C4B8 has been shown to recognize an epitope of CSPG4 in formalin-fixed paraffinembedded (FFPE) tissue sections (24). Cytoplasmic membrane staining was considered positive for CSPG4.

Immunohistochemical staining of CSPG4 protein

The immunohistochemical staining of CSPG4 protein was performed as described before (18). Following the deparaffinization and rehydration of the tumor tissue, antigen retrieval was achieved by indirectly heating the tissue in a 1mM EDTA buffer with a pH of 8.0 for 15 minutes. To prevent nonspecific binding we incubated the tissue microarray slide with a blocking buffer containing 1% Bovine Serum Albumin (BSA) and 5% normal horse serum (NHS) in Tris-Buffered Saline with Tween 20 (TBST). Incubation of the slides was performed overnight at 4 degree Celsius with the CSPG4-specific mAb D2.8.5-C4B8 (3 mg/ mL) diluted in 1% BSA and 5% NHS in TBST. Subsequent staining was achieved with the DAKO EnVision+ System-HRP (Dako North America, Inc.) and substrate diaminobenzidine (Dako North America, Inc.). Counterstaining was performed with Mayer's Hematoxylin (Lillie's Modification, Dako North America, Inc.). Finally we mounted the slides with Depex mounting medium (Electron Microscopy Sciences ®).

The staining intensity was graded by semiquantitative analysis by 2 investigators (SN, FS) where CSPG4 staining using IHC was scored using a categorical classification, 1-low expression; 2-medium expression; 3-high expression.

Generation of CAR-T cells

CAR-T cells were generated as described before (26) Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donor blood (Research Blood Components, Cambridge, MA) with Lymphoprep (Stem Cell Technologies, Cambridge, MA). On day 0, the PBMCs (1 * 106 /well) were activated in a nontreated 24-well cell culture plate (catalog 351147, Corning) precoated with 1 mg/mL CD3 (clone OKT3; Miltenyi Biotec) and 3 mg/mL CD28 antibody (clone CD28.2; BD Biosciences) in complete medium (45% RPMI1640 and 45% Click's medium (Irvine Scientific), 10% FBS, 1% Penicillin, and 1% Streptomycin (Corning)). On day 1, activated T cells were expanded by addition of IL-7 (10 ng/mL, PeproTech, Cranbury, NJ) and IL-15 (5 ng/mL, PeproTech; CAR T medium). On day 2, activated and T cells were transferred to a 24- well plate coated with RetroNectin (Takara Bio Inc., Ann Arbor, MI) containing retroviral particles of either CSPG4, or CD19 CAR construct. Following a 48 h incubation (day 4), transduced T cells were transferred to tissue culture-treated 24-well plates (# 353047; Corning) with each well containing 0.5 mL of the activated T-cell suspension (5 *105 cells/well) and 1.5 mL of fresh CAR-T medium. On day 6, an aliquot of transduced cells was analyzed for transduction efficiency, CAR-T cells in suspension were spun down, 50% supernatant was replaced with the fresh medium 50: 50 (v/v) old medium: new medium. On day 8, CAR T cells were resuspended in 2 mL of fresh CAR-T medium at 1 * 106/well to further expand cells. On day 10, cells in suspension were spun down, 50% supernatant was replaced with the fresh medium. On day 13 to 14, CAR T cells and nontransduced T (NT) cells grown at similar conditions were collected, aliquoted, and frozen for storage in a liquid nitrogen freezer for the experiments.

In vitro cell cytotoxicity assays

CSPG4 CAR T cells and target cells were co-cultured at indicated E:T ratios for 24 hrs. CAR T cells in the cell suspension were removed, and the viability of adherent target cells was quantitated by MTT assays. Mean ± SEM of cell lysis (%) of different cell populations in the chondrosarcoma cell line CS1 and SW1353 are shown. The human melanoma cell line M14 which does not express CSPG4 and M14/CSPG4 which expresses CSPG4 after stably transfected with CSPG4 plasmid DNA were used as specificity controls (27). CD19 CAR T cells were also used as a negative control since none of the target cells express CD19. The experiments were performed in triplicate and repeated 3 times, ***$p<0.001$.

Statistical analysis

Comparisons between groups were performed using Fisher's exact test. For bivariate analysis we used the log-rank test of equality across strata. This was used both for binary and categorical variables to identify factors that impact overall survival and the time to metastasis. For continuous variables as well as for binary/categorical variables we used a Cox regression analysis. Differences between CSPG4 CAR T cell-mediated cytotoxicity on different cell populations (including all E:T ratios) was detected using a chisquare test in a two-way ANOVA. STATA 12 software was used for statistical analysis purposes. p-values less than or equal to 0.05 were considered significant.

Table 1: Chondrosarcoma - Clinical and Pathological characteristics n = 76

Grade 1	**n = 17**		**Grade 2**		**n = 30**
Age, years	**Mean ± SD**	**range**	**Age**, years	**Mean ± SD**	**range**
n = 17	49 ± 14	18-69	n = 30	57 ± 16	33-83
Sex	**n**	**%**	**Sex**	**n**	**%**
male	6	35	male	15	50
female	11	65	female	15	50
Metastasis at presentation	**n**	**%**	**Metastasis at presentation**	**n**	**%**
yes	0	0	yes	0	0
no	17	100	no	30	100
Metastasis	**n**	**%**	**Metastasis**	**n**	**%**
yes	0	0	yes	6	20
no	17	100	no	24	80
Grade 3	**n = 5**		**Dediff. CS.**		**n = 24**
Age, years	**Mean ± SD**	**range**	**Age**, years	**Mean ± SD**	**range**
n = 5	56 ± 13	39-73	n = 24	59 ± 13	39-82
Sex	**n**	**%**	**Sex**	**n**	**%**
male	3	60	male	15	63
female	2	40	female	9	38

Metastasis at presentation	n	%	Metastasis at presentation	n	%
yes	0	0	yes	7	29
no	5	100	no	17	71

Metastasis	n	%	Metastasis	n	%
yes	2	40	yes	22	92
no	3	60	no	2	8,3

RESULTS

Clinical outcomes for 76 patients with chondrosarcoma

The mean and median follow-up were 5.3 ± 4.5 years (range, 0.014-19) and 4.3 years (IQR, 1.2-8.1) respectively, for the entire cohort. The mean and median follow-up for the subjects who survived long term (n= 43) were 7.8 ± 4.2 years (range, 2.0-19) and 7.1 years (IQR, 4.2-9.3) respectively. In our cohort 39 patients died due their chondrosarcoma. All subjects diagnosed with chondrosarcoma grade 1 (n=17) disease remained alive throughout the duration of the study. Seven out of the 30 subjects diagnosed with chondrosarcoma grade 2 died from their disease [mean survival time: 4.8 ± 2.3 years (range, 0.68-8.3), median survival time: 4.8 years (IQR, 4.0-6.2)] and 2 out of 5 patients with chondrosarcoma grade 3 died from their chondrosarcoma (respectively 1.3 and 3.4 years after their pathologic diagnosis). All 24 patients with a dedifferentiated chondrosarcoma died from their disease (mean survival time: 1.2 ± 1.6 years (range, 0.013-6.6), median survival time: 0.71 years (IQR, 0.38-1.3).

CSPG4 expression

Twenty-nine of the 41 (71%) conventional chondrosarcoma available for analysis show a positive (medium or high) CSPG4 expression. the largest percentage of positive samples in grade 2 (22 our of 27 tumors, 81%), followed by grade 3 (3 out of 5 tumors, 60%) and the lowest expression in grade 1 conventional chondrosarcomas (4 our of 9 tumors, 44%), (p=0.19, Figure 1).

In the dedifferentiated chondrosarcoma 3 out of 20 tumors, show positive (medium or high) CSPG4 expression (15%). The expression of CSPG4 in the dedifferentiated chondrosarcoma is less than the CSPG4 expression of conventional chondrosarcoma (P<0.001).

Of the 3 conventional chondrosarcoma metastases, 2 showed a high expression of CSPG4 and 1 metastasis showed a medium expression. All 4 dedifferentiated chondrosarcoma metastasis showed a low CSPG4 expression. Three out of the 7

enchondroma we tested showed medium or high CSPG4 expression. Representative staining results of CSPG4 are presented in Figure 2.

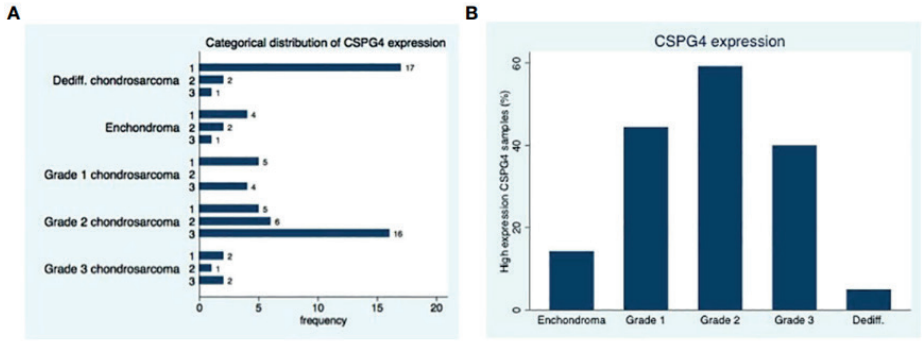

Figure 1. Distribution of CSPG4 Expression in Chondrosarcoma.

(A) CSPG4 staining using IHC was scored by 2 investigators using a categorical classification, 1-low expression; 2-medium expression; 3 high-expression. (B) The number of samples within each category that stained positive with high intensity (categorical classification 3) was plotted as a percentage of the samples in the cohort; the distribution of high intensity CSPG4 positive samples showed a unimodal distribution with the highest percentage observed in grade II. Fisher's exact p = 0.001.

Conventional chondrosarcoma, survival analysis

Using bivariate analysis, we observed a shorter time to metastasis in subjects with medium CSPG4 expression compared to the other groups in CSPG4 expression (p=0.004, Figure 3A) and there was a shorter time to death in subjects with a medium CSPG4 expression (p=0.019, Figure 3B).

We did not observe any difference in time to metastasis (p=0.68) and in time to death (p=0.95) following surgical resection of primary tumor when we compared all CSPG4-positive samples regardless of rating to CSPG4-negative samples.

Dedifferentiated chondrosarcoma, survival analysis

In the dedifferentiated chondrosarcoma cohort, we observed a shorter time to metastasis in subjects who had medium and high CSPG4 expression when compared to subjects with low or no CSPG4 expression in chondrosarcoma tissues, (p=0.048, Figure 3C). This difference was preserved when we compared all CSPG4 expressing samples (regardless of rating) with CSPG4-negative using bivariate analysis (p=0.0004).

Overall survival was shorter in subject who exhibited medium and high CSPG4 expression compared to subjects with low or no CSPG4 expression in chondrosarcoma tissues (p=0.024, Figure 3D). The 3 patients that have a medium or high CSPG4 expression have a shorter overall survival [mean survival time: 85 ± 97 days (range, 5-193), median

survival time 57 days (IQR, 5-195)] compared to the 17 patients that show no or low CSPG4 expression [mean 475 ± 638 days (range 40-2427), median survival time 319 days (IQR, 148-402)].

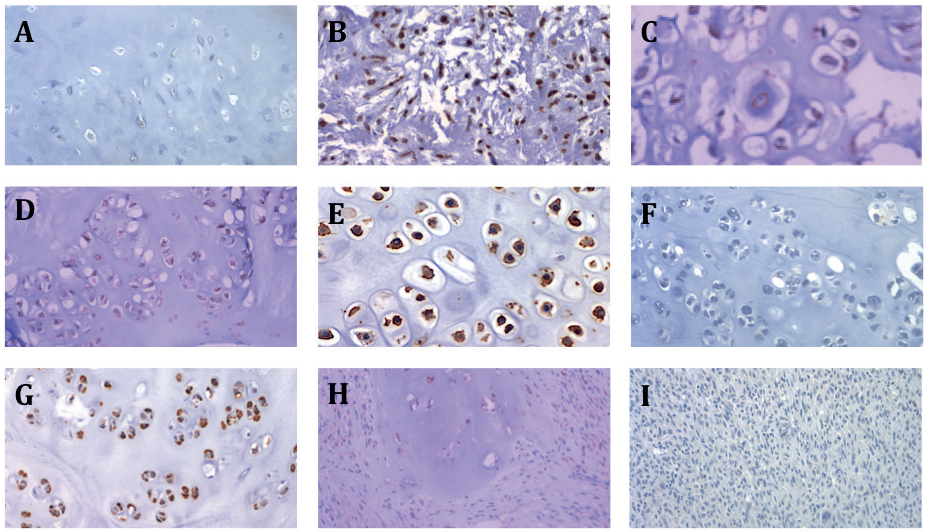

Figure 2. Representative images of CSPG4 staining of primary chondrosarcoma tumors.

(A) CSPG4 negative stain in enchondroma (200x magnification). (B) CSPG4 positive stain in grade 2 chondrosarcoma (200x magnification). (C) CSPG4 positive stain in grade 2 chondrosarcoma (400x magnification). (D) CSPG4 positive stain in grade 2 chondrosarcoma (200x magnification). (E) CSPG4 positive stain in grade 2 chondrosarcoma (400x magnification). (F) CSPG4 negative stain in grade 2 chondrosarcoma (200x magnification). (G) CSPG4 positive stain in grade 2 chondrosarcoma (200x magnification). (H) CSPG4 positive stain in dedifferentiated chondrosarcoma (200x magnification). (I) CSPG4 negative stain in dedifferentiated chondrosarcoma (200x magnification).

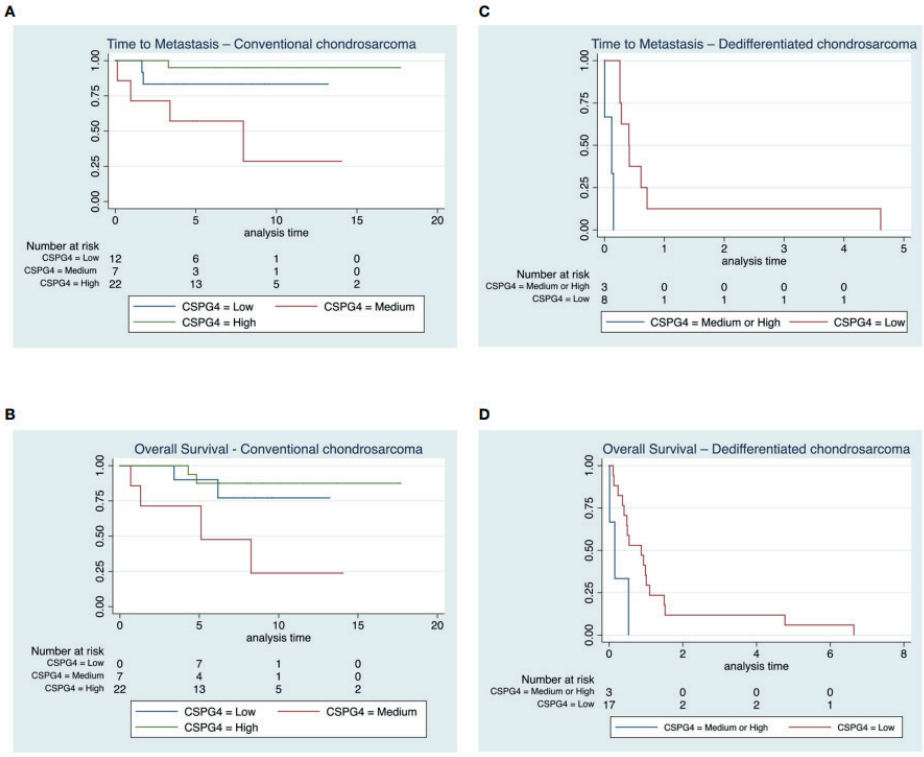

Figure 3. Time to metastasis and overall survival in conventional and dedifferentiated chondrosarcoma.

(A, B) All patients diagnosed with chondrosarcoma grade 1 (n = 17) remained alive throughout the duration of the study, 7 out of the 30 subjects diagnosed with chondrosarcoma grade 2 and 2 out of 5 patients with chondrosarcoma grade 3 died from their disease. We observed a shorter time to metastasis in subjects with medium CSPG4 expression compared to the other groups in CSPG4 expression (p = 0.004) and there was a shorter time to death in subjects with a medium CSPG4 expression (p = 0.019). (C, D) All 24 patients with a dedifferentiated chondrosarcoma died from their disease. We observed a shorter time to metastasis in subjects who had medium and high CSPG4 expression when compared to subjects with low or no CSPG4 expression in chondrosarcoma (p = 0.048). In addition overall survival was shorter in subject who exhibited medium and high CSPG4 expression compared to subjects with low or no CSPG4 expression in chondrosarcoma tissues (p = 0.024).

CSPG4 CAR T cells are effective in killing chondrosarcoma cells *in vitro*

To test whether CSPG4 antigen is an effective target for the CAR T cell-mediated killing of chondrosarcoma cells, CS1 and SW1353 chondrosarcoma cells were incubated with CSPG4- targeting CAR T cells. Subsequently we evaluated the chondrosarcoma cell survival using the MTT assay. We observed dose-dependent increases of CS1 tumor lysis of >80% in CSPG4 CAR T cell treated cells at an E:T ratio of 1:1 versus <5% tumor

lysis in the CD19 CAR T cell treated group. Similar dose-dependent increases in tumor lysis were observed in SW1353 chondrosarcoma cells, with 70% tumor lysis in CSPG4 CAR T cell treated cells at an E:T ratio of 1:1 versus <5% tumor lysis in the CD19 CAR T cell treated group (Figure 4). In both experiment set-ups there was no dose-dependent increases in M14 tumor cell killing with <2% tumor lysis at an E:T ratio of 1:1. When M14 cells were transfected to express CSPG4 antigen on its extracellular surface, a >30-fold increase in tumor lysis was observed in CSPG4- transfected M14 cells when treated with CSPG4 CAR T cells at a 1:1 E:T ratio (Figure 4).

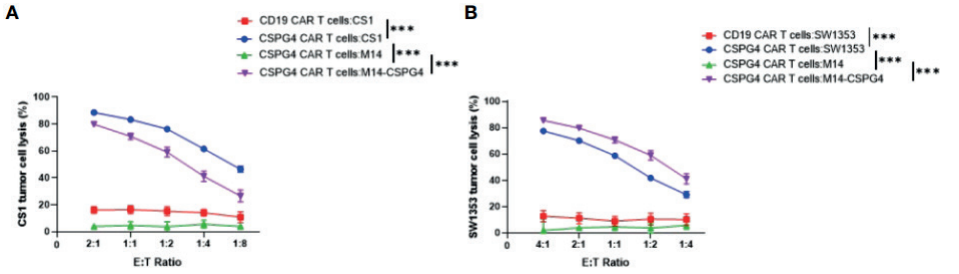

Figure 4. CSPG4-CART cells are effective in killing CSPG4-expressing chondrosarcoma cells.

CSPG4 CAR T cells and target cells were co-cultured at indicated (E) T. ratios for 24 hrs. CAR T cells in the cell suspension were removed, and the viability of adherent target cells was quantitated by MTT assays. Mean ± SEM of cell lysis (%) of different cell populations in the chondrosarcoma cell line CS1 (A) and SW1353 (B) are shown. The human melanoma cell line Ml4 which does not express CSPG4 and M14/CSPG4 which express CSPG4 after stably transfected with CSPG4 plasmid DNA were used as specificity controls (25). CD19 CAR T cells were also used as a negative control since none of the target cells express CD19. The experiments were performed in triplicate and repeated 3 times. Differences between CSPG4 CART cell-mediated cytotoxicity on different cell populations (including all E .T ratios) was detected using a chi square test in a two-way ANOVA. ***$p < 0.001$.

DISCUSSION

The current study outlines CSPG4 expression in a large series of chondrosarcoma patients showing that medium to high CSPG4 expression is associated with shorter time to metastasis and reduced overall survival in conventional and in dedifferentiated chondrosarcoma. This finding is consistent with the association of CSPG4 and overall survival in other tumor types such as breast carcinoma, ovarium carcinoma and osteosarcoma (25, 28, 29). There appears to be a biphasic pattern where CSPG4 expression increases with histological grade from enchondroma up to grade 2 and begins to decrease in grade 3 and in dedifferentiated chondrosarcoma. We interpret this observation as a possible immune escape mechanism in more aggressive lesions where subjects whose adaptive immune system generated antibodies to CSPG4 lead to an evolutionary tumor response to downregulate this

receptor. This theory is supported by findings of improved survival in melanoma patients who endogenously generated CSPG4-targeted antibodies (17, 30).

We have previously described the CSPG4 expression in chondrosarcoma and chordoma comparing the genetic profile of chondrosarcoma with other cancers (20). CSPG4 is a transmembrane proteoglycan that facilitates the interaction of cells with the extra-cellular matrix components: collagen types II, V, and VI, tenascin, laminin and fibronectin (17). CSPG4 allows cancer cells to proliferate and invade through the matrix leading to metastasis via downstream signaling pathways involving integrin-related signal transduction (31–33).

Chondrosarcoma is generally treated surgically with adequate surgical margins to control the tumor growth (34, 35). While surgery is the primary treatment of chondrosarcoma, its outcome is poor in higher-grade tumors, recurrent and metastatic tumors (35–37). Importantly, chondrosarcoma are relatively resistant to chemotherapy and radiation therapy limiting curative options for disseminated disease (5, 6).

Immunotherapy has been successfully applied clinically with many ongoing trials across many cancer types as well as various host immune machinery are currently being investigated in various passive and active immunotherapeutic protocols. One of the leading strategies is CAR T cell therapy where T cells are genetically engineered to express tumor specific antibody fragments to target and lyse cancer cells (38). Adoptive transfer of CAR T-cells has shown remarkable efficacy in treating mainly leukemias (39, 40). For solid tumors, high-antigen heterogeneity, poor tumor core infiltration, low density of tumor specific antigens as well as shared antigens between tumor and healthy cells, and an immunosuppressive tumor microenvironment pose significant challenges for CAR T cell efficacy and demonstrate the risk of on-target but off-tumor toxicities (39–41). However CSPG4 is an attractive target in cancer cells due to its well-defined role in tumor cell growth, invasion and metastasis, and its restricted expression in healthy tissue (29). Genetically engineered CSPG4-targeted CAR T cells have been shown to control tumor growth cells in vitro and in vivo in with different cell lines engrafted NSG mice (human melanoma, head and neck squamous cell carcinoma and breast carcinoma) and has been able to kill CSPG4 expressing glioblastoma cancer stem cells (42, 43).

Importantly, we observed efficient killing of CSPG4-expressing chondrosarcoma cellsin vitro when incubated with CSPG4-targeted CAR T cells demonstrating that CSPG4-targeted CAR T cell immunotherapy may be an effective adjuvant therapy in CSPG4-positive conventional and dedifferentiated chondrosarcoma tumors. This strategy might be of particular relevance since our group and others have shown that HLA class I expression is frequently reduced in conventional chondrosarcoma,

limiting immunotherapeutic strategies targeting machinery upstream of tumor antigen crosspresentation in the absence of induced HLA class I stimulation (7–9). In contrast, the CAR T cell therapy is independent of HLA class I antigen expression and might give therapeutic options in chondrosarcoma tumors with defective HLA class I expression.

Furthermore, we can increase CSPG4 expression in CS1 and SW1353 chondrosarcoma cells with one subclinical dose of irradiation (10 Gy) (Supplemental Figure 1) suggesting that CSPG4 CAR T efficacy may further be augmented when combined with irradiation.

Taken together, these findings support the role of CSPG4 as a promoter of disease and therefore as a clinically relevant target in patients with chondrosarcoma. The results of this study demonstrate that CSPG4 directed therapies may be applicable to chondrosarcomas with over 70% of patients in our cohort demonstrating moderate to high expression in conventional chondrosarcoma. CSPG4 directed therapies might be particularly attractive in cases of high-grade conventional chondrosarcoma, local recurrence, and metastasis. Our study has 2 main limitations due to use of paraffin embedded tumors from one hospital and our three-tier system scoring approach for stained tumors that may have caused us to miss subtle differences in staining patterns. In addition the sample size in the survival analysis is small, especially for the dedifferentiated chondrosarcoma, limiting the possibility to account for potential confounders. Future studies might investigate CSPG4 protein expression in an independent patient series determining whether our data are generalizable. Nonetheless, our findings are consistent with published reports on CSPG4 as a prognostic marker in cancer and provide a rationale for future studies to investigate the effectiveness of CSPG4 directed therapy in chondrosarcoma *in vivo.*

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PART III

Outcomes after oncological resection around the shoulder

5

Chapter 5

Outcome After Reconstruction of the Proximal Humerus for Tumor Resection: A Systematic Review

Clin Orthop Relat Res. 2014

T. Teunis, S.P.F.T. Nota, F.J. Hornicek, J.H. Schwab, S.A. Lozano-Calderon

ABSTRACT

Background

Tumors of the appendicular skeleton commonly affect the proximal humerus, but there is no consensus regarding the best reconstructive technique after proximal humerus resection for tumors of the shoulder.

Questions/purposes

We wished to perform a systematic review to determine which surgical reconstruction offers the (1) best functional outcome as measured by the Musculoskeletal Tumor Society (MSTS) score, (2) longest construct survival, and (3) lowest complication rate after proximal humerus resection for malignant or aggressive benign tumors of the shoulder.

Methods

We searched the literature up to June 1, 2013, from MEDLINE, EMBASE, and the Cochrane Library. Only studies reporting results in English, Dutch, or German and with follow ups of 80% or more of the patients at a minimum of 2 years were included. Twenty-nine studies with 693 patients met our criteria, seven studies (24%) were level of evidence III and the remainder were level IV. Studies reported on reconstruction with prostheses (n = 17), osteoarticular allografts (n = 10), and allograft-prosthesis composites (n = 11). Owing to substantial heterogeneity and bias, we narratively report our results.

Results

Functional scores in prosthesis studies ranged from 61% to 77% (10 studies, 141 patients), from 50% to 78% (eight studies, 84 patients) in osteoarticular graft studies, and from 57% to 91% (10 studies, 141 patients) in allograft-prosthesis composite studies. Implant survival ranged from 0.38 to 1.0 in the prosthesis group (341 patients), 0.33 to 1.0 in the osteoarticular allograft group (143 patients), and 0.33 to 1.0 in allograft-prosthesis group (132 patients). Overall complications per patient varied between 0.045 and 0.85 in the prosthesis group, 0 and 1.5 in the osteoarticular graft group, and 0.19 and 0.79 in the prosthesis-composite graft group. We observed a higher fracture rate for osteoarticular allografts, but other specific complication rates were similar.

Conclusions

Owing to the limitations of our systematic review, we found that allograft-prosthesis composites and prostheses seem to have similar functional outcome and survival rates, and both seem to avoid fractures that are observed with osteoarticular allografts. Further collaboration in the field of surgical oncology, using randomized controlled trials, is required to establish the superiority of any particular treatment.

INTRODUCTION

The proximal humerus is the second most common site of all osseous sarcomas and the third most common site for osteosarcoma. Although osteosarcomas and Ewing's sarcomas occur characteristically in teenagers and young adults, chondrosarcomas occur in older individuals [18]. Reconstruction of the shoulder after resection of a malignant or a benign locally aggressive primary bone tumor of the proximal humerus poses the challenging problem of associated bone loss. In addition, an adequate tumor margin implies partial resection of the deltoid musculature and joint capsule and occasionally the rotator cuff, axillary nerve, glenoid, or the scapula.

There is no consensus regarding the best reconstructive technique after proximal humerus resection. Principal treatment approaches in use today include arthroplasty prostheses, osteoarticular allografts, and allograft-prosthesis composites [38, 58]. Moreover, several autologous grafts (fibula, scapular crest, or clavicle [5, 8, 47]) have been described. Because autologous grafts often are used in conjunction with a shoulder arthrodesis, prostheses, osteoarticular allografts, and allograft-prosthesis composites are the only reconstructions allowing for a mobile glenohumeral joint. Although all of these approaches are in use, and there are some situations where only one approach might be appropriate for a particular patient, there are many scenarios in which all are potential options. However, because there are no prospective or randomized trials, it is difficult to know which approach is best in terms of functional outcome, implant survivorship, or complications.

This review aims to identify which surgical reconstruction (1) offers the best functional outcome measured by the Musculoskeletal Tumor Society (MSTS) score [20], and (2) has the longest survival rate, and (3) lowest complication rate after proximal humerus resection for tumors of the shoulder.

MATERIALS AND METHODS

Article Selection

On June 1, 2013, we searched PUBMED, EMBASE, and the Cochrane Library using the search string for title and abstract: (humerus OR shoulder OR ''upper limb'' OR ''upper extremity'') AND (neoplasm* OR tumor* OR tumour* OR malign* OR sarcoma* OR cancer*) AND (prosthe* OR autograft OR allograft OR fusion OR flail joint OR Tikhoff linberg OR arthrodesis OR clavicula pro humero OR graft OR forequarter amputation). This search yielded 524 results from PubMed, 548 from EMBASE, and three from the Cochrane Library. Two reviewers (SAL-C, TT) independently examined the citation information for each result from the databases for relevant studies; subsequently, two independent reviewers screened the full texts (TT, SPFTN); they also scanned

the reference lists of the included articles for additional studies that met the inclusion criteria (Fig. 1). Inclusion criteria were determined as follows: functional outcome after proximal humerus resection for any malignant or benign locally aggressive tumor, a minimum 2-year followup, and English, Dutch, or German language publication. We excluded cohorts with less than three patients and case reports, preclinical studies, meeting abstracts, indiscernible proximal humerus patient cohorts, inadequate outcome reporting, studies with more than 20% of patients lost to followup, and salvage procedures. In case of overlapping patient cohorts [36, 48, 51, 52], we included the study reporting on the largest cohort [36, 52]. In one of three studies [23, 37, 43], after correspondence with the author, we included the smaller cohort with a more comprehensive functional outcome [23].

Two independent reviewers (TT, SPFTN) critically appraised the included studies using predetermined criteria, and data were extracted with standardized sheets. Discordant judgments were resolved by consensus discussion between the two reviewers. Critical appraisal criteria included disclosure, selection of patients, outcome reporting and assessment, baseline display, and postoperative care. These criteria are based on the relevant literature [14, 28, 38, 49, 58]. Our review is registered with PROSPERO (registration number CRD42013005626) [54, 55].

Outcome Measurements

The following data were extracted from the selected articles: author, year of publication, institute, study type, construct included, number of patients, age, follow-up, patients lost to follow-up, nature of the tumor (benign or malignant), metastasis, implant survival, complications, and MSTS score. We considered the following reconstructive techniques: prostheses, osteoarticular allografts, allograft-prosthesis composites, fibula autografts, scapular crest flaps, clavicle pro humero reconstruction, flail joint, and amputation.

Functional Outcome

In case of missing MSTS score standard deviations or baseline characteristics, we contacted the corresponding authors; of the contacted authors of 19 studies [1, 6, 9, 12, 16, 17, 19, 25, 27, 31, 36, 43, 45, 47, 50, 56, 60–62], 13 replied [1, 6, 9, 16, 17, 27, 31, 36, 43, 45, 50, 56, 61] and seven were able to provide us with the requested additional data [6, 9, 17, 27, 36, 45, 56]. We regarded autoclaved autograft and allograft bone combined with a prosthesis as allograft-prosthesis composites. Osteoblastomas and giant cell tumors are scored as benign locally aggressive tumors with their unpredictable behavior and rare incidences of metastasis [13, 26].

The mean MSTS score with its SD was extracted or, if appropriate, calculated from individual patient data. In two cases, scores were calculated differently: in one

instance, we used the lowest possible MSTS score reflecting ''poor'', ''good'', and ''excellent'' outcomes as reported by the authors, possibly underestimating the functional results [30]. In another study we estimated the SD by computing the two and four missing values resulting in the largest SD [44].

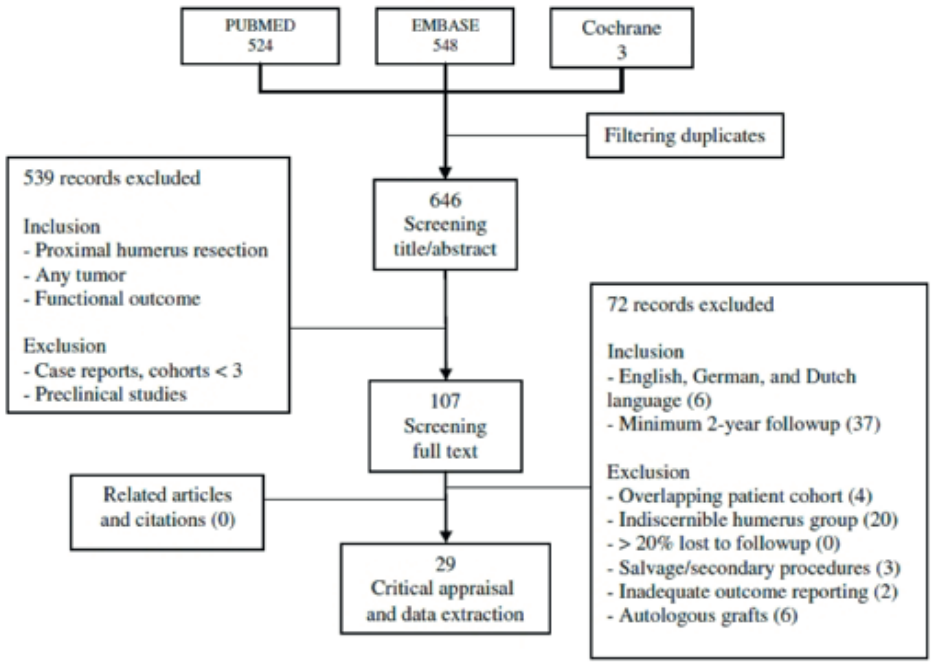

Fig. 1 The flowchart shows our literature search and selection of relevant articles. The last search was performed on June 1, 2013

Implant Survival

We considered total or partial removal of the reconstruction a failure. Kaplan-Meier implant survival rates were extracted at the 5-year end point.

Complications

We evaluated the following complications: deep infections, fracture, subluxation, dislocation, proximal migration, component loosening, nonunion, and permanent nerve deficit.

Study Characteristics

Articles on amputation either focused on patient survival or included multiple disorders other than proximal humerus malignancies. No article on clavicula pro humero satisfied our inclusion criteria. Scapular crest and fibula grafts used various

proximal fixation methods: plate [5, 8, 60] or wire [34], wire and tendon [59], suture [36], or none at all [7, 21]. In addition, in three studies, it was reported that an allograft was used to strut the fibular grafts [7, 21, 60], and Kumar et al. [34] reported that in one instance, an additional scapular crest graft was used. Arthroplasty prostheses, osteoarticular allografts, and allograft-prosthesis composites are comparable in the sense that they allow for reconstructions of a mobile glenohumeral joint and have similar indications, therefore only those reconstructions were included in our final analysis.

Approximately 1/4 of the studies were level III evidence and the remainder were level IV. Critical appraisal shows only 55% of the studies reported eligibility criteria, sources, and methods of selection of participants, possibly resulting in selection bias. As only 14% reported what complications would be collected before their actual data collection and only one study used objective, independent outcome reporting, outcome bias cannot be excluded (Fig. 2) (Appendix 1, Supplemental material is available with the online version of CORR). Asymmetry of the funnel plots seems to reflect the clinical and methodologic heterogeneity rather than publication bias (Fig. 3) [28].

Study Population

For this review, a total of 693 patients were included with a mean age per study ranging from 9 to 57 years. The percentage of primary malignancies varied between 23% and 100%, secondary malignancies from 0% to 77% (0% in 21 studies), and benign tumors from 0% to 75% (0% in 18 studies; Table 1).

Analysis

Because of heterogeneity and sensitivity to bias when not including randomized controlled trials we narratively reported our results. MSTS scores are reported as percentages; survival and complications are reported as proportions to the included patients.

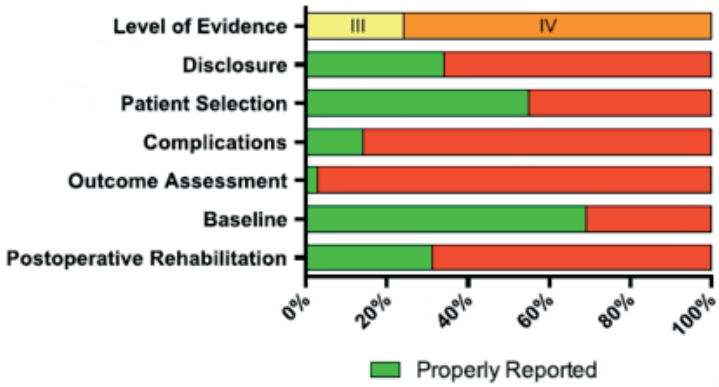

Fig. 2 Low reporting of patient and complication selection and outcome assessment increase the risk for selection and outcome bias.

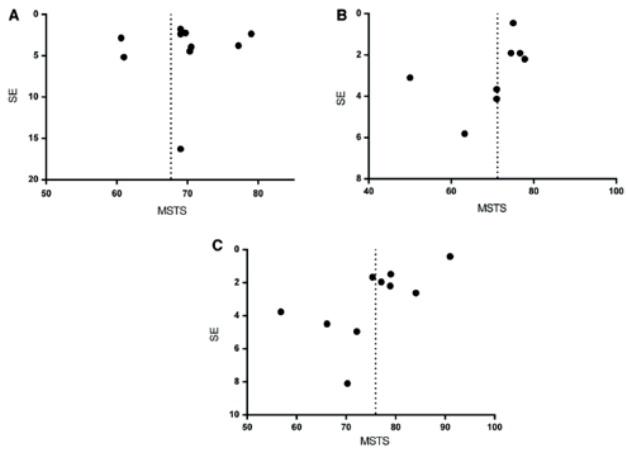

Fig. 3A–C The funnel plots show MSTS scores and standard errors for (A) prosthesis studies, (B) osteoarticular allograft studies, and (C) allograft-prosthesis composite studies. Asymmetry seems to reflect clinical and methodologic heterogeneity rather than publication bias

RESULTS

MSTS Scores

Twenty-four studies reported the MSTS scores for 28 reconstructive methods for a total of 398 patients. Functional score in prosthesis studies ranged from 61% to 77% (10 studies, 141 patients); osteoarticular grafts from 50% to 78% (eight studies, 84 patients); allograft-prosthesis composites from 57% to 91% (10 studies, 141 patients; Table 2).

Implant Survival

Implant survival and complications were calculated for 616 patients from 29 studies. Implant survival ranged from 0.38 to 1.0 in the prosthesis group (341 patients); 0.33 to 1.0 in the osteoarticular allograft group (143 patients), and 0.33 to 1.0 in allograft-prosthesis group (132 patients; Table 3).

Complications

Overall complications per patient varied between 0.045 and 0.85 in the prosthesis group, 0 and 1.5 in the osteoarticular graft group, and 0.19 and 0.79 in the prosthesis-composite graft group. In particular, the fracture rate varied among groups; proportions ranged from 0.05 to 0.17 with prostheses and 0 to 0.67 with osteoarticular grafts and composite-prosthesis allografts. However, when only including studies with more than 10 patients the fracture rates ranged from 0 to 0.05 in the prosthesis group, 0.08 to 0.62 in the osteoarticular allograft group, and 0 and 0.06 in the allograft-prosthesis composite group (Table 3). Other complications were similar between constructs (Appendix 2, Supplemental material is available with the online version of CORR).

Table 1. Characteristics of the included studies

Study	Type	Construct	Patients	Mean age (years)	Follow-up (years/range)	Malignant	Metastasis	Benign
Burrows et al. [11]	CS	P	7	47	13 (4-24)	57%	14%	29%
Bos et al. [10]	CS	P	15	29	6 (2-16)	67%	0%	33%
Malawer et al. [35]	CS	P	29	NR	4 (2-12) ^	100%	0%	0%
O'Connor et al. [44]	RC	P	11	35 *	5 (2-12)	NR	NR	NR
Meller et al. [41]	CS	P	8	34	3 (2-8)	88%	13%	0%
Voggenreiter et al. [57]	CS	P	19	49	5 (0-11)	89%	11%	0%
Fuhrmann et al. [22]	CS	P	22	57	4 (2-7)	23%	77%	0%
Wittig et al. [61]	CS	P	23	18^	10 (2-20)	100%	0%	0%
Kumar et al. (1)	CS	P	100	34	9 (2-20)	78%	18%	4%
Mayilvahanan et al. [39]	CS	P	57	28	6 (2-15)	65%	9%	26%
Kitagawa et al. [32]	CS	P	10	49	2 (0-9)	80%	0%	20%
Sharma et al. [50]	CS	P	21	41*	5 (0-13)*	100%	0%	0%
Potter et al. [45]	RC	P	16	54	8 (2-18)^	38%	56%	6%
Ioannou et al. [29]	CS	P	12	46	6 (4-9)	83%	17%	0%
Manfrini et al. [36]	RC	P	25	11	11 (SD 8)	100%	0%	0%
van de Sande et al. [56]	RC	P	14	44	17 (3-25)	57%	21%	21%
Wang et al. [60]	RC	P	25	19*	7 (3-16)	100%	0%	0%
Gebhardt et al. [23]	CS	OA	20	42*	16 (2-28)*	65%	5%	30%
Aho et al. [3]	CS	OA	4	NR	6 (3-20)*	25%	0%	75%
Alman et al. [4]	CS	OA	3	13	5 (3-7)	100%	0%	0%
O'Connor et al. [44]	RC	OA	8	35*	5 (2-12)	NR	NR	NR
Probyn et al. [46]	CS	OA	11	34	4 (2-7)	100%	0%	0%

Study	Type	Construct	Patients	Mean age (years)	Follow-up (years/range)	Malignant	Metastasis	Benign
Getty & Peabody [24]	CS	OA	16	35	4 (1-11)	88%	0%	13%
DeGroot et al. [15]	CS	OA	31	30	6 (2-12)	55%	10%	35%
Potter et al. [45]	RC	OA	17	37	8 (2-18)^	59%	24%	18%
Manfrini et al. [36]	RC	OA	3	12	3 (NR)	100%	0%	0%
van de Sande et al. [56]	RC	OA	13	33	17 (3-25)	69%	0%	31%
Aponte-Tinao et al. [6]	RC	OA	21	32*	5 (1-20)	81%	0%	19%
Jensen & Johnston [30]	CS	APC	14	43	5 (2-12)	93%	0%	7%
Suk et al. [52]	CS	APC	6	26	4 (3-5)	100%	0%	0%
Black et al. [9]	CS	APC	6	41	5 (2-6)	83%	17%	0%
Potter et al. [45]	RC	APC	16	56	8 (2-18)^	50%	38%	13%
Abdeen et al. (1)	CS	APC	36	23*	5 (0-11)	89%	8%	3%
Moran & Stalley [42]	CS	APC	11	22	6 (2-9)	100%	0%	0%
Manfrini et al. [36]	RC	APC	3	9	4 (NR)	100%	0%	0%
van de Sande et al. [56]	RC	APC	10	34	17 (3-25)	70%	10%	20%
Wang et al. [60]	RC	APC	14	19*	7 (3-16)	100%	0%	0%
Aponte-Tinao et al. [6]	RC	APC	16	42	5 (1-20)	100%	0%	0%

CS = case series; RC = retrospective cohort; P = prosthesis; OA = osteoarticular allograft; APC = allograft-prosthesis composite; NR = not reported; * cohort, ^ median.

Table 2. Functional outcome

Study	Construction	Sample size	MSTS score (%)	Standard deviation
O'Connor et al. [44]	Prosthesis	6	61	13
Meller et al. [41]	Prosthesis	6	71	10
Voggenreiter et al. [57]	Prosthesis	10	70	14
Fuhrmann et al. [22]	Prosthesis	22	61	13
Kumar et al. (1)	Prosthesis	30	79	13
Kitagawa et al. [32]	Prosthesis	3	69	28
Potter et al. [45]	Prosthesis	16	69	9
Manfrini et al. [36]	Prosthesis	21	70	10
van De Sande et al. [56]	Prosthesis	10	77	12
Wang et al. [60]	Prosthesis	17	69	7
O'Connor et al. [44]	Allograft	6	71	10
Probyn et al. [46]	Allograft	10	50	10
Getty & Peabody [24]	Allograft	8	63	16
DeGroot et al. [15]	Allograft	23	74	9.1
Potter et al. [45]	Allograft	12	71	13
Manfrini et al. [36]	Allograft	3	78	3.8
van De Sande et al. [56]	Allograft	6	77	4.7
Aponte-Tinao et al. [6]	Allograft	16	75	1.8
Jensen & Johnston [30]	APC	14	77	7.3
Suk et al. [52]	APC	6	57	9.2
Black et al. [9]	APC	4	70	16
Potter et al. [45]	APC	15	79	5.7
Abdeen et al. (1)	APC	34	91	2.4
Moran & Stalley [42]	APC	8	66	13
Manfrini et al. [36]	APC	3	79	3.8
van de Sande et al. [56]	APC	7	72	13
Wang et al. [60]	APC	10	75	5.3
Aponte-Tinao et al. [6]	APC	13	84	9.4

MSTS = Musculoskeletal Tumor Society; APC = allograft-prosthesis composite

Table 3. Implant survival and complications

Study	Construct	Sample size	Implant survival	Proportion	Complications	Proportion	Fractures	Proportion
Burrows et al. [11]	Prosthesis	6	5	0.83	5	0.83	1	0.17
Bos et al. [10]	Prosthesis	13	5	0.38	11	0.85	2	0.15
Malawer et al. [35]	Prosthesis	29	26	0.9	NR	NR	NR	NR
O'Connor et al. [44]	Prosthesis	11	7	0.63	9	0.82	1	0.091
Meller et al. [41]	Prosthesis	8	8	1.0	NR	NR	NR	NR
Voggenreiter et al. [57]	Prosthesis	19	17	0.89	4	0.21	2	0.11
Fuhrmann et al. [22]	Prosthesis	22	22	1.0	1	0.045	0	0
Wittig et al. [61]	Prosthesis	15	15	1.0	2	0.13	1	0.067
Kumar et al. (1)	Prosthesis	45	39	0.87	10	0.22	0	0
Mayilvahanan et al. [39]	Prosthesis	55	50	0.91	16	0.29	3	0.055
Kitagawa et al. [32]	Prosthesis	5	4	0.8	3	0.60	0	0
Sharma et al. [50]	Prosthesis	21	14	0.67	3	0.14	0	0
Potter et al. [45]	Prosthesis	16	16	1.0	5	0.31	0	0
Ioannou et al. [29]	Prosthesis	12	NR	NR	2	0.17	0	0
Manfrini et al. [36]	Prosthesis	25	20	0.80	15	0.60	0	0
van de Sande et al. [56]	Prosthesis	14	14	1.0	7	0.50	0	0
Wang et al. [60]	Prosthesis	25	23	0.92	22	0.88	0	0
Gebhardt et al. [23]	Allograft	20	16	0.80	10	0.50	5	0.25
Aho et al. [3]	Allograft	4	2	0.50	5	1.3	2	0.50
Alman et al. [4]	Allograft	3	1	0.33	3	1.0	2	0.67
O'Connor et al. [44]	Allograft	8	6	0.75	4	0.50	4	0.50
Probyn et al. [46]	Allograft	10	4	0.40	10	1.0	4	0.40

Study	Construct	Sample size	Implant survival	Proportion	Complications	Proportion	Fractures	Proportion
Getty & Peabody [24]	Allograft	13	8	0.61	20	1.5	8	0.62
DeGroot et al. [15]	Allograft	31	23	0.74	16	0.52	11	0.35
Potter et al. [45]	Allograft	17	12	0.71	14	0.82	9	0.53
Manfrini et al. [36]	Allograft	3	3	1.0	0	0	0	0
van de Sande et al. [56]	Allograft	13	8	0.62	11	0.85	1	0.077
Aponte-Tinao et al. [6]	Allograft	21	16	0.76	5	0.24	5	0.24
Jensen & Johnston [30]	APC	14	14	1.0	3	0.21	0	0
Suk et al. [52]	APC	6	5	0.83	2	0.33	1	0.17
Black et al. [9]	APC	6	5	0.83	2	0.33	0	0
Potter et al. [45]	APC	16	15	0.94	7	0.44	1	0.063
Abdeen et al. (1)	APC	36	33	0.92	10	0.28	1	0.028
Moran & Stalley [42]	APC	11	11	1.0	6	0.55	0	0
Manfrini et al. [36]	APC	3	1	0.33	2	0.67	2	0.67
van de Sande et al. [56]	APC	10	9	0.90	15	1.5	2	0.20
Wang et al. [60]	APC	14	12	0.86	11	0.79	0	0
Aponte-Tinao et al. [6]	APC	16	13	0.81	3	0.19	1	0.063

MSTS = Musculoskeletal Tumor Society; APC = allograft-prosthesis composite; NR = not reported

DISCUSSION

Tumors of the appendicular skeleton commonly affect the proximal humerus, and complete resection impedes shoulder function. As there is no consensus regarding the best reconstructive technique after proximal humerus resection, reviewing the literature in the absence of quality randomized prospective trials might offer some insight into the best reconstructive option. We aimed to identify which surgical reconstruction offers (1) the best functional outcome measured by the MSTS score, and (2) has the longest survival rate and (3) lowest complication rate after proximal humerus resection. Because of the limited literature available we were able to compare only arthroplasty prostheses, osteoarticular allografts, and allograft-prosthesis composites.

This study has some limitations. Because this is a review of nonrandomized studies, there is an increased risk of selection bias, variation in the way in which confounding is considered in the analysis, and greater risk of other biases. All of these biases would tend to increase apparent benefits of treatments and deemphasize the problems and complications associated with these treatments. Additionally, the most commonly used score in the papers reviewed in this investigation used the MSTS score, resulting in a possibly overstated physician-perceived function, instead of a true patient-reported outcome. The MSTS score is not a validated tool and may not adequately reflect upper extremity function, as it mainly measures impairment instead of activity limitation. Moreover, the MSTS score is a physician-rated instrument and a couple studies stress its subsequent limitations [2, 53].

Owing to the limited information reported, the current results do not allow for subgroup analysis on different clinical scenarios (eg, tumor type and stage, age, soft tissue resection, or radiographic findings). This impedes the predictive value of our results for specific patients. We include previously missing, and therefore unpublished, MSTS score standard deviations and baseline characteristics from seven studies that were not verified by peer review [6, 9, 17, 27, 36, 45, 56].

Although allograft-prosthesis composites resulted in the largest range in MSTS scores, the majority of the scores for all three constructs were between 60% and 79%, making them seem largely comparable in functional outcome.

Implant survival looks similar between the three constructs.

The number of overall complications per patient seems greater in the osteoarticular allograft group (range, 0–1.5 versus 0.045–0.85 in the prosthesis group and 0.19–0.79 in the allograft-prosthesis composite group). The increased complication rate seems to be based on higher osteoarticular allograft fracture rates, as all other specific complications were comparable. Fracture rates between the osteoarticular allograft

group and allograft-prosthesis composite group might appear similar (both 0–0.67); however, the upper limits with allograft-prosthesis composite fractures are based on relatively small studies. When comparing only studies with more than 10 patients, the allograft-prosthesis composite fracture rates (0–0.06) are comparable to those of the prosthesis (0–0.05).

Performance of a (superior) randomized controlled trial is hindered by several practical difficulties; one is the necessity of a surgeon or group of surgeons being able to confidently perform highly specialized operations such fibular transplantation, including vascular microsurgery and adequate tumor resection. Another major problem is the number of patients presenting with oncologic conditions requiring resection of the proximal humerus. A power analysis of three reconstructive methods, assuming a difference in MSTS score of 10%, shows a required sample size of 969 patients (alpha 0.05; power 0.8; G*Power 3.1.7). Because the cohort studies in our review reported an average of 1.6 to 5.3 patients per year [6, 32, 36, 44, 45, 56, 57, 60, 61], a randomized controlled trial would be an elongated, if not impossible, endeavor at any institution. Nonetheless, according to the IDEAL recommendations [40] to evaluate surgical innovations, all surgical procedures for proximal humerus replacement are far from validated. The next step would be assessment by inclusion of large groups of patients using standardized surgeries, postoperative care, and outcome reporting. Because a randomized controlled trial might be difficult to conduct, several other options are available, for example, matching procedures or an expertise-based randomized trial. The latter involves a study in which patients are randomly assigned by a third party to surgeons, who then treat their patients with their preferred intervention [40]. This design prevents the exigency of different highly specialized surgeons at one institution and does not require any surgeon to abandon his or her preferred method.

The limitations of the literature we surveyed suggest strongly to us that multicenter studies are warranted if we are to establish the optimal treatment for oncologic replacement of the complex shoulder. A prospective database including patients from specialized treatment centers would be an important first step. Strengths of our systematic review of the available retrospective literature were its restriction to follow-up of at least 2 years and restriction to studies that accounted for 80% or greater of the patients they included. Conscious of the limitations of our systematic review in coming to firm conclusions, allograft-prosthesis composites and prostheses probably are indistinguishable based on the literature, and both seem to avoid the problem of fracture that is observed with osteoarticular allografts.

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6

Chapter 6

Functional Outcomes and Complications After Oncologic Reconstruction of the Proximal Humerus

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ABSTRACT

Background

No consensus exists on the best method of articular reconstruction in patients who require proximal humerus resection for the management of primary bone sarcomas, soft-tissue sarcomas extending into the bone, benign and locally aggressive primary bone tumors, and metastatic disease.

Methods

We identified patients from two institutions who underwent wide resection of the proximal humerus along with oncologic reconstruction using osteoarticular allografts (OAs), endoprostheses, or allograft-prosthesis composites. We prospectively collected functional outcomes and retrospectively assessed complications and implant survival.

Results

A total of 150 patients were included in this study. The average Disabilities of the Arm, Shoulder and Hand questionnaire score was 26 for 25 patients, of which we gathered their functional data, with no differences in physical function among the three constructional methods according to the Disabilities of the Arm, Shoulder and Hand questionnaire, upper extremity Toronto Extremity Salvage Score, upper extremity Musculoskeletal Tumor Society, and Patient-Reported Outcomes Measurement Information System scores. Overall, the survival rate of the prosthesis was >50%. A trend was noted for a higher risk of failure in the OA group secondary to the allograft fracture.

Discussion

All three articular oncologic shoulder reconstructions were comparable in terms of function. This large series confirms a higher fracture rate in OAs, which explains the observed higher revision rate and apparent lower survival rate in this subgroup.

BACKGROUND

Following the knee and proximal femur, the shoulder is the most common location for all primary bone sarcomas (1). Usually, treatment of these tumors involves wide resection with subsequent reconstructive surgery. The main types of reconstruction include shoulder arthrodesis and functional or mobile reconstruction with osteoarticular allografts (OAs), endoprostheses (EPs), or allograft-prosthesis composites (APCs) (2). These modalities of treatment may also be applied to metastatic disease. No consensus exists on the best reconstruction method. Limited literature exists that demonstrates potential differences in functional outcomes, complications, and survival of the construct to guide both clinicians and patients in deciding on the reconstruction technique.

A recent review of the literature from our group included 24 publications

evaluating reconstructions of the proximal humerus in a total of 398 patients (3). Because of the low epidemiologic level of the published literature and poor quality in outcome assessment reporting, we could not identify any difference in final function supporting one method of reconstruction over the other.

This investigation is a prospective collection of functional outcomes in patients from two institutions who underwent oncologic reconstruction of the proximal humerus after wide resection of primary malignant or benign and locally aggressive osseous tumors, malignant soft-tissue tumors extending into the bone, locally aggressive soft tissue tumors, or metastatic disease of the proximal humerus. Our primary null hypothesis was that there was no difference in the Disabilities of the Arm, Shoulder and Hand questionnaire (DASH) score among OA, EP, and APC reconstructions of the proximal humerus. The secondary study goal was to investigate differences in complication rates and implant survival among these methods of reconstruction.

METHODS

Study Design

Under an institutional review board-approved protocol, we identified all patients aged ≥18 years at the time of the study who underwent wide resection and reconstruction of the proximal humerus for primary bone sarcomas, benign and locally aggressive bone tumors, soft-tissue sarcomas, lymphoma, or metastatic lesions of the proximal humerus at both institutions.

This cohort was established by an automated systematic query for the word “humer” and screening of pathology reports of both institutions between 1990 and 2013. These automated searches resulted in 1,183 and 406 patients, respectively. Subsequently,

we selected patients who underwent humeral reconstruction with OAs, EPs, or APCs, resulting in 93 and 39 patients, respectively, from the two databases. In addition, a surgical orthopaedic oncology registry from one of the institutions was searched for additional eligible patients covering a time frame from 1976 up to 1990 (n = 18). This resulted in a final cohort of 150 patients.

Perioperative Information

Beforehand, selected outcome variables were retrieved from the patients' digital medical files. We collected the following data: type of reconstruction, previous surgeries on the shoulder, pathologic fracture before reconstruction, involved side, and surgery for metastatic disease. We used the Malawer classification for humeral resections to assess the bones and soft tissues that were resected. Malawer et al (4) classify resection based on six types of anatomic resections in the shoulder area. Resection types 1, 4, 5, and 6 contain the proximal humerus; type 1 is an intra-articular proximal humeral resection and type 5 is an extra-articular humeral and glenoid resection. Letter A indicates conservation of the abductor muscles, whereas resection is indicated by letter B. We also registered if part of the glenoid, scapula, clavicle, deltoid muscle, and rotator cuff was resected as part of the procedure. In addition, we retrieved the size of humerus bone resection. Finally, we checked whether patients were given a spica cast as part of their postoperative management (see Table 2, Supplemental Digital Content 1, http://links.lww.com/JAAOS/A95).

Demographic and Oncologic Data

From medical records, we extracted the following data: sex, age at the time of diagnosis and resection, primary diagnosis, metastatic disease at the initial diagnosis, and occurrence of a pathologic fracture at the initial clinical presentation (see Tables 1 and 2, Supplemental Digital Content 1 and 2, http://links.lww.com/JAAOS/A95 and http://links.lww.com/JAAOS/A96).

Functional Data Cohort

To determine the vital status of 150 patients in our cohort, we consulted, apart from the medical records, the Social Security death index and registered 56 patients who were alive. Of these 56 patients, 4 patients were not able to communicate in English and, therefore, were not approached. The other 52 patients were invited by letter to participate in the study to fulfill selected functional outcome questionnaires to assess their upper extremity function. After sending out the letter, we were informed that 8 of the 52 patients were no longer alive, although this finding was not recorded. Of the remaining 44 patients, 13 were lost to follow-up, 1 refused to participate, and 3 agreed to participate but never filled the questionnaires. Finally, 2 patients were not able to fill the questionnaires at the time of assessment because of planned and/

or current hospital admittance, which resulted in available functional data from 25 patients (see Table 5, Supplemental Digital Content 3, http://links.lww.com/JAAOS/A97).

These 25 patients differed from the other 125 patients according to several variables: a higher percentage (84% versus 16%) of primary versus metastatic disease (P<0.001), a larger size of proximal humerus osseous resection (15 versus 11 cm; P = 0.0099), and a longer median time of follow-up (7.1 versus 1.5 years; P<0.001) (see Appendix, Supplemental Digital Content 7, http://links.lww.com/JAAOS/A101).

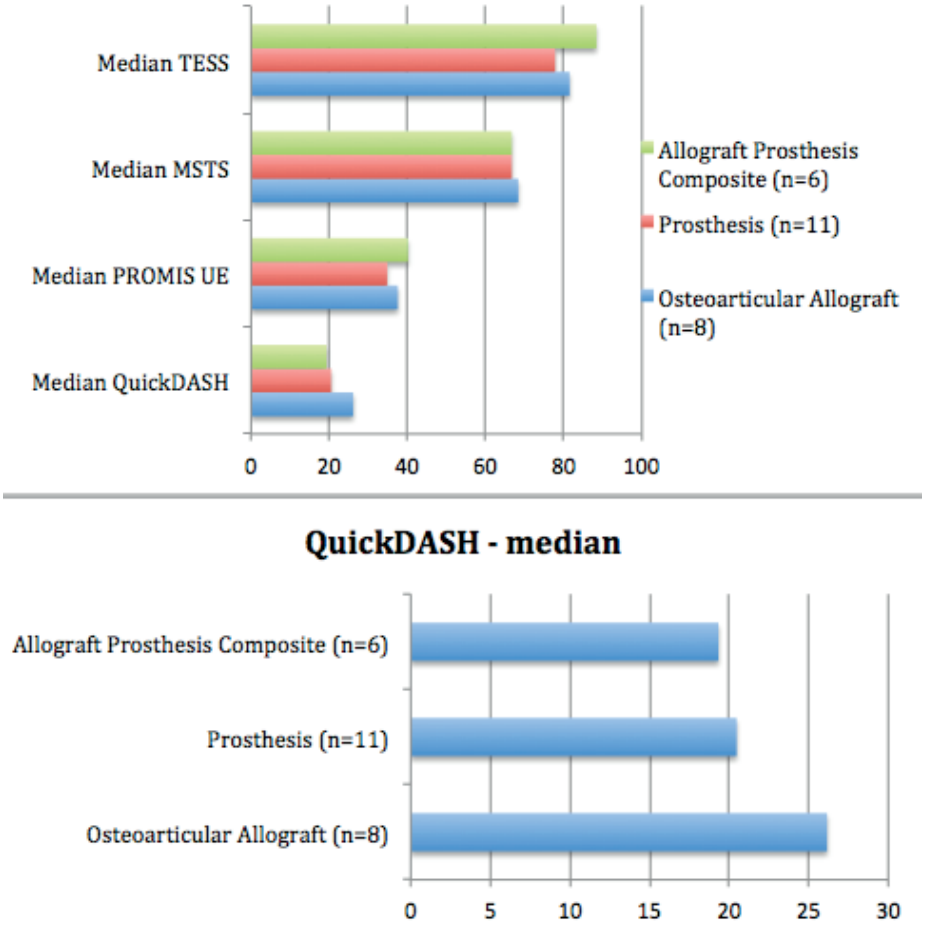

Figure 1. A and B, Graphs comparing functional outcomes among constructs. MSTS = Musculoskeletal Tumor Society, PROMIS UE = Patient-Reported Outcomes Measurement Information System Upper Extremity, QuickDASH = Disabilities of the Arm, Shoulder and Hand questionnaire, TESS = Toronto Extremity Salvage Score.

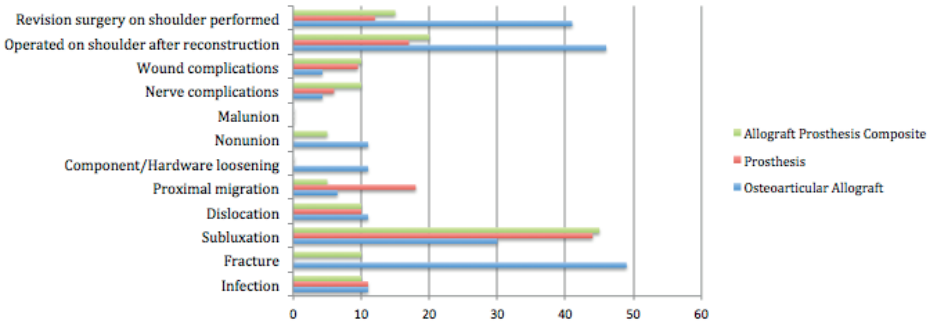

Figure 2. Graph showing the percentage of complications.

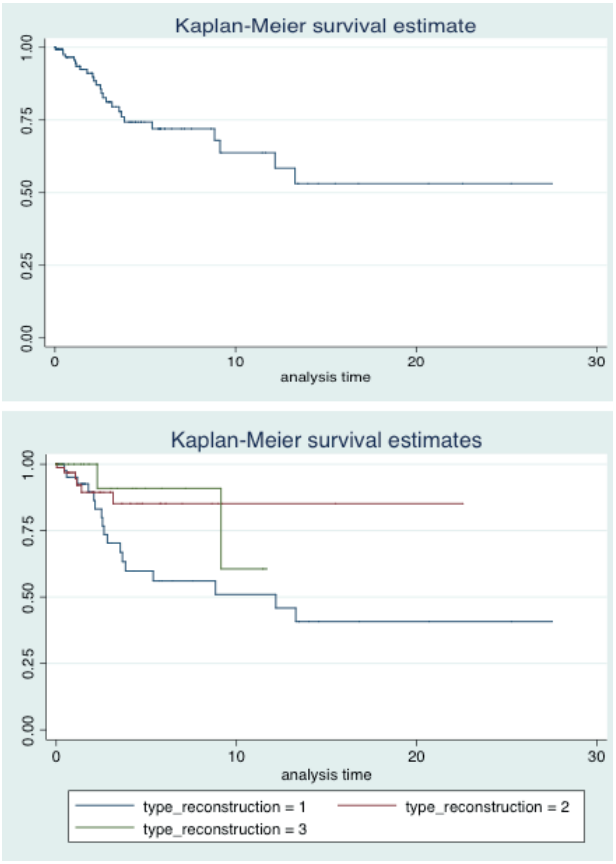

Figure 3 A and B, Graphs showing Kaplan-Meier curves displaying overall survival and comparing constructs.

Functional Outcome Questionnaires

Patients implied consent to participate in the study by completing the questionnaires, or they decided on participation by returning the distributed refusal/participation card. Questionnaires were completed online through Research Electronic Data Capture Tools (5) over the phone or in written format. Four different questionnaires were used to assess the patients' upper extremity function. These questionnaires were the short version of the DASH (QuickDASH) (6), the Computer Adaptive Test of the Patient-Reported Outcomes Measurement Information System (PROMIS), Physical Function Upper Extremity (7), the upper extremity Toronto Extremity Salvage Score (TESS) (8), and the upper extremity Musculoskeletal Tumor Society (MSTS) rating scale (9), which we converted to a patient-dependent assessment version instead of the original physician-rated system. Our main outcome is the QuickDASH which provides a disability score ranging from zero to 100, where a higher score represents higher disability. The average DASH score for the American population is 10 ± 15 (10). For the PROMIS Physical Function Upper Extremity, 50 is the mean in the US general population, with an SD of 10; a higher score indicates higher physical function. The TESS ranges from zero to 100% of the maximum score, with a higher score indicating less disability. The MSTS total score ranges from zero to 30, where a higher score indicates less disability. It can be converted to a percentage of the maximum score as well (as in this study).

Complications

We gathered information about the occurrence of the following postoperative complications: surgical site infection, fracture, subluxation or dislocation of the reconstruction, proximal migration of the humerus, component loosening, nonunion, malunion, nerve complications, wound complications, and the total number of revision surgeries performed on the shoulder differentiating between revision surgeries and other surgical procedures in which the index reconstruction was left in place (see Table 3, Supplemental Digital Content 4, http://links.lww.com/JAAOS/A98)

Prosthesis Survival Analysis

Revision surgery of the prosthesis was used as an end point for our survival analysis. The surgical procedure was considered a revision surgery if the reconstruction was (partially or entirely) removed from the patient, except if the reason for revision surgery was tumor recurrence (see Table 4, Supplemental Digital Content 5, http://links.lww.com/JAAOS/A99).

Statistical Analysis

Because of nonparametric distribution of the data, Kruskal-Wallis oneway analysis of variance was used for multiple comparisons, whereas the Mann-Whitney U test

was used to test differences in the median of numeric variables between the two groups. The Fisher exact test was used to investigate the significance of differences in contingency tables. Correlations were displayed with the Spearman rank correlation test.

Kaplan-Meier curves were used to display implant survival statistics. In bivariate analysis, the log-rank test of equality across strata was used for binary and categoric variables to identify factors influencing time to revision surgery. Cox regression analysis was used for the continuous variables and to establish a multivariable model evaluating the risk of construct failure. We regarded a value of $P<0.05$ as significant.

Demographics

A total of 150 patients were included in this study; approximately half were male (49%). The average age was 53 ± 19 years at the time of reconstruction. Seventy-three patients underwent surgery the right-sided humerus (49%). Most of the patients (89/150; 59%) underwent surgery for metastatic disease. Most of the patients (84/150; 56%) underwent reconstruction with EPs, followed by OAs (46/150; 31%) and APCs (20/150; 13%). The average follow-up was 5.0 ± 6.6 years (range, 1 to 40 months), where the median follow-up was 2.3 years (interquartile range [IQR], 0.42 to 6.5) (see Table 1, Supplemental Digital Content 2, http://links.lww.com/JAAOS/A96).

The most common diagnosis in terms of primary sarcomas of bone included osteosarcoma, 21 patients (14%); chondrosarcoma, 18 patients (12%); and Ewing's sarcoma, 4 patients (2.7%). As mentioned, the most common diagnosis was metastatic disease; the most common primary tumors included renal cell carcinoma in 29 patients (19%), breast carcinoma in 15 patients (10%), and metastatic lung carcinoma in 13 patients (8.7%) (see Table 6, Supplemental Digital Content 6, http://links.lww.com/JAAOS/A100).

RESULTS

Functional Analysis

We observed no difference among the three constructional methods in physical function as measured with the QuickDASH. The average QuickDASH score was 26 ± 16. The QuickDASH negatively correlated with the PROMIS Upper Extremity, MSTS, and TESS, with correlation coefficients of 20.64 ($P = 0.0014$), 20.70 ($P <0.001$), and 20.67 ($P <0.001$), respectively.

The median QuickDASH score for the OA group (n = 8) was 26 (IQR, 22 to 35), for the EP group (n = 11) 20 (IQR, 14 to 45), and for the APC group (n = 6) 19 (IQR, 11 to 30) (Figure 1).

We did not find any difference in upper extremity function measured by PROMIS Upper Extremity, TESS, or MSTS scores (see Table 5, Supplemental Digital Content 3, http://links.lww.com/JAAOS/A97).

Complications

Fractures (49% [OA] versus 4.8% [EP] versus 10% [APC]; P<0.001), component loosening (11% [OA] versus 1.2% [EP] versus zero [APC]; P = 0.032), and nonunion (11% [OA] versus zero [EP] versus 5.0% [APC]; P = 0.007) of the reconstruction were more common in the OA group. No differences exist in postoperative infection, subluxation and dislocation, proximal humerus migration, or nerve and wound complications among the different reconstruction techniques (see Table 3, Supplemental Digital Content 4, http://links. lww.com/JAAOS/A98) (Figure 2).

Prosthesis Survival

Overall, the survival rate of the prosthesis was >50%, with a failure rate of 0.047 per year and a 25th-percentile survival time of 3.8 years (Figure 2). There seems to be a trend toward a higher risk of failure in the OA group (failure rate per year: 0.064 [OA] versus 0.031 [EP] versus 0.026 [APC]; P = 0.070) (Figure 3). If the OA group is compared with the other two reconstruction methods combined, it is also associated with a higher failure rate (P = 0.022). Other factors associated with higher failure in bivariate analysis are (partial) removal of the deltoid muscle, infection, fracture, dislocation, component loosening, wound and nerve complications, and a younger age at the time of reconstruction (see Table 4, Supplemental Digital Content 5, http://links.lww.com/JAAOS/A99).

A total of 32 revision surgeries were performed, 7 of which were performed for tumor progression, whereas 25 were for failure of reconstruction. The etiology of the 25 revision surgeries included fractures in 10 patients (40%), infection in 5 (20%), both infection and fracture in 2 (8%), infection combined with hardware failure in 1 (4%), osteoarthritis with pain in 1 (4%), pain with limited function in 1 (4%), osteonecrosis with fracture in 1 (4%), dislocated prosthesis in 2 (8%), dislocation, fracture, and infection in 1 (4%), and resorption of the humeral head in 1 (4%).

The following 32 revision surgeries were performed: 12 of the reconstructions were revised in a singlestage manner to an EP, 4 into an APC, and 2 into an OA. Five patients received a two-stage revision with an antibiotic spacer. Only two of these underwent subsequent reconstruction with an APC or an EP. Two patients received a resection arthroplasty only, and one patient was partially revised by replacing the humeral head. In addition, six patients required an amputation for revision surgery.

A bivariate analysis to identify potential predictors of construct failure was performed using multiple demographic, peri-, intra-, and postoperative variables

(excluding the complications as predictors of failure). This analysis demonstrated the number of revision surgeries (hazard ratio [HR], 2.7; P = 0.019), use of OAs versus the other two methods (HR, 2.6; P = 0.027), intact deltoid (HR, 0.45; P = 0.045), and younger age (HR, 0.97; P = 0.004) as predictors of failure. A multivariable regression analysis, including solely the type of reconstructions and the number of revision surgeries, identified the number of revision surgeries before a potential revision (HR, 2.6; P = 0.030) and the use of an OA (HR, 2.4; P = 0.071) as the strongest predictors of reconstruction failure (P = 0.024). When age at the time of reconstruction and the type of reconstructions are included in a multivariable model, age is a predictor of failure (HR, 0.97; P = 0.020), where the influence of the use of an OA (HR, 1.3; P = 0.64) and APC (HR, 0.45; P = 0.36) is uncertain.

DISCUSSION AND SUMMARY

In this study, we investigate the functional outcomes of patients who underwent oncologic reconstruction of the proximal humerus. Subsequently, we monitored the potential complications after reconstruction and prosthesis survival. We did not identify any difference in physical function among the three constructional methods as measured with the QuickDASH in our relatively small cohort.

In a previously published review, it is reported that the functional outcomes of these three reconstruction methods are largely comparable when assessing 24 studies (3 reconstructive methods, 398 patients). Musculoskeletal Tumor Society scores range from 50% to 87% for the OA, from 61% to 77% for the EP, and from 57% to 91% for the APC between studies (3). However, these outcomes are measured with physician-rated, nonvalidated, MSTS scores that potentially lead to overrating in contrast to our choice to let the patients fill the converted MSTS questionnaires. The ranges reported in this review of the literature are comparable with those of our functional cohort (n = 21; mean, 64%; range, 53% to 80%; IQR, 73% to 87%).

We encourage other groups to follow this similar model and instruments to facilitate the combination of raw data that may help elucidate the superiority of one construct over the others.

It is traditionally thought that the use of OAs is at higher risk of infection, nonunions, delayed unions, and fractures. Conversely, EPs are perceived as more susceptible to subluxation, dislocation, proximal migration, and decreased abduction. The two largest series that evaluate shoulder OAs are from Gebhardt et al (11) and Aponte-Tinao et al (12). Gebhardt et al (11) evaluated OAs in 20 patients, showing 7 patients with an allorgraft fracture. Their function was rated as “good” as measured with the MSTS. Aponte-Tinao et al (12) showed comparable results in a group of 21 patients (five fractures) and a 5-year survival rate of the allograft of 79% and a 10-year survival rate of 69%.

Allograft-prosthesis composite reconstruction has the combined advantages of the OA and EP: bone preservation and soft-tissue reattachment possibilities combined with the rigidity and articular robustness of the prosthesis. However, drawbacks are combined as well. Abdeen et al (13) looked at 36 consecutive patients who underwent APC reconstruction. They show a survival rate of 88% at 10-year follow-up.

Conversely, the analysis of this series, the largest in terms of OA and EP numbers, demonstrates that the infection rate is comparable among the three reconstruction methods, as it is the rate of subluxation, dislocation, and proximal humeral migration.

In terms of survival, we identified that the number of revision surgeries and the use of allografts are potential predictors of failure. However, the effect of the type of reconstruction is uncertain when age is included in a model together with the type of reconstruction. Models including more variables were not considered possible because of the limited amount of failures. In a recent literature review (3), the reported implant survival rate for the OA is 0.33 to 1.0, for the EP 0.38 to 1.0, and for the APC 0.33 to 1.0, and therefore, we concluded that implant survival is similar. In comparison, the implant survival rate found in our study is 0.41 for the OA, 0.12 for the EP, and 0.15 for the APC.

Limitations of the study should be taken into account when interpreting the results of our investigation. First, this is a retrospective study including the data from two large academic hospitals representing the experience of a small number of surgeons and may therefore not reflect the results of others. Second, restrictions exist concerning the comparability of three reconstruction techniques, which may partially arise from the variability in the indications for surgery. These minor deviations in indications are partially reflected in differences in age, the percentage of metastatic disease, the amount of spica cast revalidation used, and the size of the resection between groups. These differences and the comparability between constructs are presumably influenced mostly by the relatively different indications and treatments between metastatic and primary disease.

In addition, significant differences exist between the functional data group and the rest of the cohort, and therefore, we can only draw conclusions about function in this group, which may not reflect patients treated for metastatic disease.

In conclusion, reconstruction of the proximal humerus after oncologic resections is challenging. Articular methods of reconstruction such as the OA, EP, and APC are comparable in terms of function in our series. Complication rates are also comparable in terms of infection, subluxation, dislocation, proximal migration of the humerus, and delayed union. This large series confirms a higher fracture rate in OAs than their counterparts. This higher fracture rate explains the observed higher revision rate and apparent lower survival rate compared with endoprostheses or APCs.

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7

Chapter 7

Functional and oncological outcome of surgical resection of the clavicle and scapula for primary chondrosarcoma.

Musculoskelet Surg. 2016

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ABSTRACT

Purpose

The scapula is a relatively common site for chondrosarcoma to develop in contrary to the clavicle, which is rarely affected by these tumors. The aim of this study is to determine the functional and oncological outcome for patients treated operatively for scapular or clavicular chondrosarcoma.

Methods

In this single-center retrospective study, we included a sample of 20 patients that received the diagnosis of a primary chondrosarcoma of the scapula or clavicle. Of the surviving patients, the functional function was assessed using the DASH and the PROMIS Physical Function—Upper Extremity. Patients were longitudinally tracked for their oncological outcome.

Results

All patients were followed for at least 2 years or until death. The mean age of the cohort was 47 years. Eighteen patients suffered from a chondrosarcoma of the scapula, and in 2 patients, the tumor was located in the clavicle. Metastasis, local recurrence and a higher tumor grade were all associated with a decreased overall survival. For the patients with a chondrosarcoma of the scapula, the average DASH score was 16 ± 16 and the mean PROMIS Physical Function—Upper Extremity score was 48 ± 10. Patients with both an intact rotator cuff and glenoid had a better physical function.

Conclusions

Upper extremity function after (partial) scapulectomy varied depending on whether the glenoid was spared and whether a functioning shoulder abductor remained. When the resection spared these structures, then excellent functional outcomes were reported. Oncologic outcomes depended upon the grade of the tumor and whether local recurrence and metastases occurred.

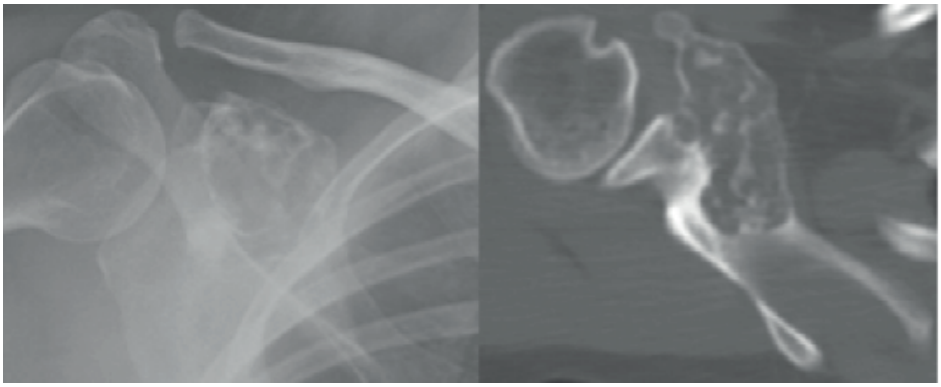
INTRODUCTION

Chondrosarcoma is the second most common primary malignant bone tumor [1–4] of which approximately 20% develop in the shoulder girdle [5, 6]. Within the shoulder, the scapula is a relatively common site for these tumors to develop [7–9] in contrary to the clavicle which is rarely affected by chondrosarcoma [6, 10]. Unni and Inwards [6] describe 1073 cases of chondrosarcomas in his review of which 55 tumors were located in the scapula and only 6 were located in the clavicle. Surgical resection is the standard of care for conventional chondrosarcoma since chemotherapy and radiation therapy have limited effect [2, 7, 9].

Although the functional outcome has been of interest in some studies, there is more literature available regarding the oncological outcomes of chondrosarcoma in the scapula. The majority of the functional data that are available assess patients' functional status from a physicians standpoint instead of reporting patients-reported outcomes [11]. The data regarding chondrosarcoma of the clavicle are limited to reports of a few cases [6, 7, 12, 13]. As Griffin et al. [5] report, there have been some contradictions in the literature concerning the oncological outcomes of chondrosarcoma in the scapula. While one study states that the rates of local recurrence and metastasis are high [14], others claim the contrary [3, 7, 15].

The aim of this study is to determine the functional and oncological outcome for patients treated operatively for scapular or clavicular chondrosarcoma.

(a)



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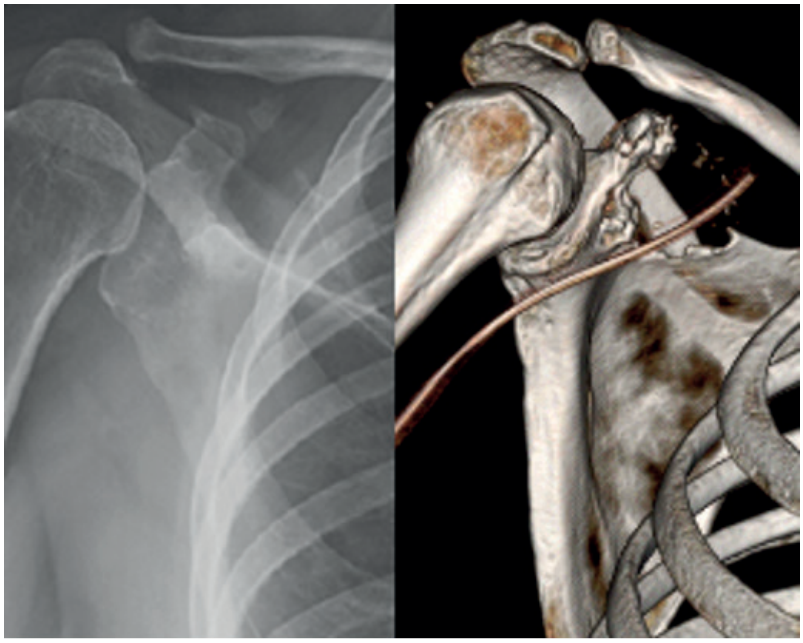

Fig. 1 a Posterior–anterior radiograph (left) and axial computed tomography (right) of a grade 1 chondrosarcoma of the scapula before surgical resection (patient 1). **b** Posterior–anterior radiograph (left) and three-dimensional computed tomography (right) of the scapula after surgical resection of a grade 1 chondrosarcoma (patient 1)

MATERIALS AND METHODS

Study design

After receiving Institutional Review Board (IRB) approval for this single-center retrospective study, a sample of 20 patients that received the diagnosis of a primary chondrosarcoma of the scapula or clavicle was identified from our departmental database. The identification was performed by manually chart reviewing medical records after a systematic electronic query of all pathology reports of surgically treated patients for a primary orthopedic malignancy between 1993 and 2011 in our institution. We only included patients initially treated at our institution, and patients with secondary invasion of the scapula or clavicle from a chondrosarcoma were not included.

All surviving patients were approached by mail and/or telephone to participate in this study. Between March and June 2014, we assessed the present upper extremity physical function of the patients who agreed to participate. We managed and collected the study data using the online Assessment Center data collection tool. Of the 13 surviving patients, 1 patient declined to participate due to his high age and 2 patients were lost to follow-up resulting in the functional outcome data of 10 patients.

Outcome measures

We extracted the following data from the patients' medical records: age at the time of diagnosis, sex, metastasis at presentation, location of tumor, size of tumor, grade of tumor, metastasis, location of metastasis, time to metastasis, local recurrence, time to local recurrence, extra osseous extension of the tumor, surgical margins (mm), type of surgery/reconstruction, vital status, time to death, cause of death, radiotherapy and/or chemotherapy used. Patients were longitudinally tracked from the histological confirmed date of diagnosis until the latest follow-up or death.

We divided the oncological resections into the following 2 main groups: claviculectomy and scapulectomy (partial and total). Furthermore, we monitored if the glenoid fossa and the rotator cuff were intact following the oncological resection.

The patient's physical function was assessed with 2 questionnaires: the Disabilities of the Arm, Hand and Shoulder questionnaire (DASH) [16] and the Computer Adaptive Test of the Patient Reported Outcomes Measurement Information System (PROMIS), Physical Function—Upper Extremity version [17]. A higher DASH score represents higher disability (range 0–100). The mean score in the American population for the DASH is 10 ± 15 [18]. Higher PROMIS scores represent a better function with a score of 50 representing the norm in the US population and every 10 points a standard deviation from the norm.

Statistical analysis

Normality of all continuous data was tested using the Shapiro–Wilk test. Nonparametric tests were used since all our data showed a nonparametric distribution. We used the Mann–Whitney U test in bivariate analysis to compare two groups of continuous data. Log-rank test was to compare the survival distribution of 2 samples. Cox proportional hazard models were used to test the influence of continuous variables on survival.

Table 1: Baseline characteristics

Patient number	Sex	Age	Location	Volume (cm3)	Grade	Extra osseous extension	Closest margin (mm)	Metastasis	Local Recurrence	Follow-up years	Vital status
1	Female	51	Scapula	*45**	1	0	.	0	0	6.7	Alive - NED
2	Male	40	Scapula	245	3	1	2	1	1	10	Dead - Cancer
3	Female	29	Scapula	45	2	0	3	0	0	18	Alive - NED
4	Male	31	Scapula	1470	1	1	0	1	0	18	Alive - NED
5	Male	77	Scapula	142	2	1	2	0	0	18	Alive - NED
6	Male	63	Scapula	360	2	1	<1	1	1	1.5	Dead - Cancer
7	Female	70	Scapula	792	dd	1	<1	1	0	0.31	Dead - Cancer
8	Female	37	Clavicle	69	1	1	<1	0	0	5.8	Alive - NED
9	Male	39	Scapula	36	2	1	<1	0	0	16	Alive - NED
10	Male	50	Scapula	224	2	1	<1	0	0	15	Alive - NED
11	Male	49	Scapula	87	1	1	0	0	0	12	Alive - NED
12	Female	33	Scapula	*5.8⁺*	1	1	<1	0	0	11	Alive - NED
13	Female	38	Scapula	5888	2	1	1.5	0	0	2.0	Alive - NED
14	Female	46	Clavicle	81	1	1	<1	0	0	7.8	Alive - NED
15	Female	40	Scapula	101	2	1	4	1	1	3.2	Dead - Cancer
16	Female	41	Scapula	624	1	1	4	0	0	1.7	Dead - Cancer^
17	Female	66	Scapula	176	3	1	1	1	1	3.7	Dead - Cancer
18	Male	55	Scapula	202	2	0	10	0	0	21	Alive - NED
19	Male	44	Scapula	600	dd	1	<1	1	1	0.93	Dead - Cancer
20	Male	38	Scapula	10	2	0	0	0	0	12	Alive - NED

dd = dedifferententiated chondrosarcoma,* = volume largest bone part of aggregate containing the tumor, ⁺ = largest dimension
^ = Ollier disease ; developed dedifferentiated chondrosarcoma in the pelvis

Table 2: Surgical resection and functional outcome

Patient number	Surgery	Graft/ prosthesis	Glenoid intact	Rotator cuff intact	DASH outcome measure	PROMIS Physical Function Upper Extremity
1	Partial scapulectomy	0	0*	0	28	35
2	Partial scapulectomy & Distal claviculectomy	0	0	1	n/a	n/a
3	Partial scapulectomy	0	1	1	1.7	56
4	Partial scapulectomy	0	0*	0	10	47
5	Partial scapulectomy	OA pelvic allograft	0	0	n/a	n/a
6	Partial scapulectomy & Distal claviculectomy	0	1	0	n/a	n/a
7	Total scapulectomy	0	0	0	n/a	n/a
8	Proximal claviculectomy	0	1	1	0.83	56
9	Partial scapulectomy	OA scapula allograft	0	1^	22	37
10	Scapulectomy & Distal claviculectomy	OA pelvic allograft & PH prosthesis	0	0	19	56
11	Partial scapulectomy	0	0*	0	n/a	n/a
12	Partial scapulectomy	0	1	0	47	37
13	Partial scapulectomy & Distal claviculectomy	0	0	0	n/a	n/a
14	Total claviculectomy	0	1	1	3.3~	56~
15	Partial scapulectomy	0	1	0	n/a	n/a
16	Total scapulectomy	0	0	0	n/a	n/a
17	Total scapulectomy	0	0	0	n/a	n/a
18	Partial scapulectomy	0	1	1	0.83	56
19	Total scapulectomy & Distal claviculectomy	0	0	0	n/a	n/a
20	Partial scapulectomy	0	1	1	0	56

* partial resection, ^ reconstructed, OA= Osteoarticular, PH = Proximal Humerus, ~patient filled out questionnaires twice (DASH = 4.2, PROMIS UE = 48)

Table 3: Surgical resection and functional outcome

Patient number	Surgery	Glenoid intact	Rotator cuff intact	DASH outcome measure	PROMIS Physical Function Upper Extremity
8	Proximal claviculectomy	1	1	0.83	56
14	Total claviculectomy	1	1	3.3	56
3	Partial scapulectomy	1	1	1.7	56
18	Partial scapulectomy	1	1	0.83	56
20	Partial scapulectomy	1	1	0	56
9	Partial scapulectomy	0	1	22	37
12	Partial scapulectomy	1	0	47	37
1	Partial scapulectomy	0	0	28	35
4	Partial scapulectomy	0	0	10	47
10	Scapulectomy & Distal claviculectomy	0	0	19	56

Table 4: Comparing functional outcome for the scapular resections

	DASH			PROMIS PF-UE		
	Mean	SD	P-value	Mean	SD	P-value
Partial/total scapulectomy, n=8	16	16		48	10	
Glenoid intact						
yes, n=4	12	23	0.25	51	9.9	0.28
no, n=4	20	7.3		44	10	
Rotator cuff intact						
yes, n=4	6.0	10	0.083	52	9.6	0.17
no, n=4	26	16		44	10	
Rotator cuff & glenoid intact						
yes, n=3	0.83	0.83	0.025	56	0	0.057
no, n=5	25	14		42	9.1	

PH = Physical Function, UE = Upper Extremity, SD = Standard Deviation

RESULTS

Twenty patients were included in our study. All patients were followed for at least 2 years or until death with a mean follow-up of 12 ± 5.7 years (range 2.0–21) for the surviving patients. The mean age of the cohort was 47 ± 13 years (range 29–77) at the time of diagnosis, and half of the patients (10 patients) were male. Eighteen patients suffered from a chondrosarcoma of the scapula, and in 2 patients, the tumor was located in the clavicle. An example case of a scapula chondrosarcoma is provided in Fig. 1. The average tumor size was 589 ± 1333 cm2 (range 10–5888 cm2) (Table 1).

Eight patients underwent more operations for their disease than the primary resection; 1 patient had a surgical margin positive for chondrosarcoma resulting in a reoperation, 1 patient underwent a wound debridement for a surgical site infection, 2 patients were re operated due to hardware failure, and 4 patients were operated for advancing local disease of which 2 patients had metastasis as well.

Oncological outcome

Seven patients were histologically diagnosed with a grade 1 chondrosarcoma, 9 patients were diagnosed with a grade 2 chondrosarcoma, 2 patients were diagnosed with a grade 3 chondrosarcoma, and 2 patients were diagnosed with a dedifferentiated chondrosarcoma (Table 1). One patient had metastasis at the time of diagnosis (patient 7). Five patients had a local recurrence of their disease after an average time of 2.5 ± 2.9 years (range 0.54–7.2 years). Seven patients developed metastasis after an average time of 2.0 ± 2.8 years (range -0.027 to 7.7 years). All metastasis were located in the lungs. Seven patients died of cancer; 6 patients died of a chondrosarcoma of the scapula; and 1 patient, diagnosed with Ollier disease, died due to a primary developed dedifferentiated chondrosarcoma in the pelvis within 2 years of the diagnosis of chondrosarcoma of the scapula (patient 16). The mean time to death after diagnosis was 3.1 ± 3.3 years (range 0.31–10). Seven patients received radiotherapy, and 3 patients were treated with chemotherapy for their disease.

The overall survival was 13 out of 20 patients in our cohort, and approximately 60% of the patients are still alive after 10 years (Fig. 2). Sex ($P = 0.48$), age ($P = 0.21$), the volume of the tumor ($P = 0.96$), a positive surgical margin ($P = 0.19$) and the length of the tumorfree margin ($P = 0.96$) were not associated with longer overall survival. Metastasis ($P < 0.001$), local recurrence ($P < 0.001$) and a higher tumor grade ($P < 0.001$; Fig. 3) were all associated with a decreased overall survival. More than 75% of the patients with a grade 1 or grade 2 tumors are alive after 10 years. The patients with a grade 3 or dedifferentiated tumors have a worse survival, and they all died from their disease in 10 years (Fig. 3).

Functional outcome

Of the 10 patients who filled out their upper extremity function questionnaires, 2 patients were treated with a claviculectomy alone and 8 patients received a (partial) scapulectomy. Naturally the 2 patients with only a claviculectomy had an intact glenoid and rotator cuff. Four out of 8 (50%) patients with a (partial) scapulectomy had an intact glenoid, 4 out of 8 (50%) had an intact rotator cuff, and 3 out of 8 patients (38%) had both an intact rotator cuff and glenoid after surgical resection of the tumor in their scapula (Tables 2, 3). For the patients with a chondrosarcoma of the scapula, the average DASH score was 16 ± 16 and the mean PROMIS Physical Function—Upper Extremity score was 48 ± 10. The 2 patients with a claviculectomy had a DASH score of, respectively, 0.83 and 3.3 and both a PROMIS score of 56. Patients treated for a chondrosarcoma in their scapula with both an intact rotator cuff and glenoid had a better physical function as measured with DASH (P = 0.025) and PROMIS (P = 0.057; Table 4).

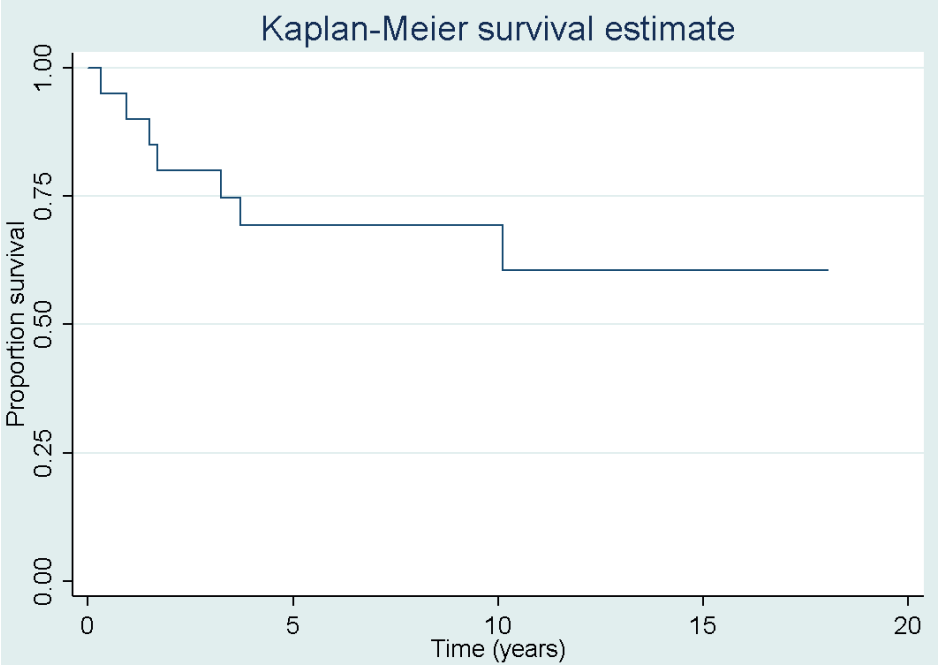

Fig. 2 Kaplan–Meier survival estimates, overall survival

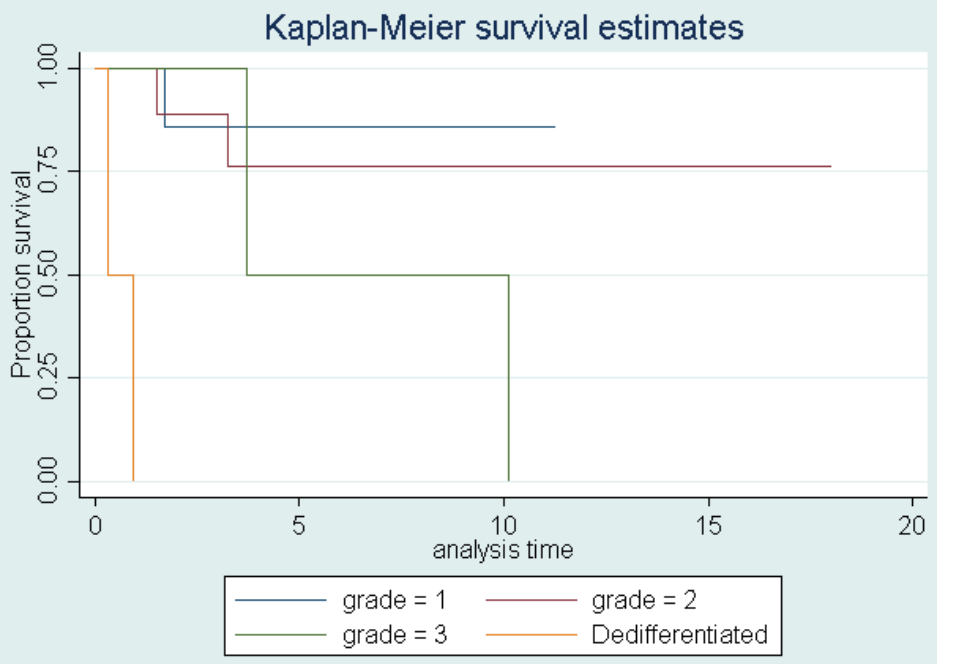

Fig. 3 Kaplan–Meier survival estimates, survival per grade

DISCUSSION

We report the functional and oncological outcome of patients treated with surgical resection with a chondrosarcoma of the clavicle or scapula. We report the functional outcome of 10 of these patients that we divided into 2 groups: claviculectomy and (partial) scapulectomy. The average DASH score was 16, and the mean PROMIS Physical Function—Upper Extremity score was 48 for the patients with a (partial) scapulectomy. We reported a significant better function for patients who have their rotator cuff and glenoid intact compared to the patient who have those structures affected. The fact that the upper extremity disability of this cohort and the reported disability of the general population are relatively comparable is remarkable and implies a good physical function following surgical resection. However, the individual variation in outcome should be noticed.

Gibbons et al. conclude, in a series of 14 patients with a variety of bone tumors in the scapula requiring partial scapulectomy, that an excellent functional status can be maintained when the glenohumeral joint is preserved. However, they state that the rotator cuff can be removed without much functional loss (if the deltoid is

reconstructed) which cannot be concluded from our study [19]. Schwab et al. [20] showed the superior function in patients with a retained functioning deltoid in patients receiving a scapular prosthesis which is in line with our finding of the impact of resection of essential anatomical structures in the shoulder girdle. Griffin et al. [5] showed a statistical significant difference in both physician-rated and patients-rated functional outcome between patients treated with a partial scapulectomy and patients treated with a total scapulectomy favoring the former. These findings provide evidence that size of the resection and the involved anatomical structures in these parts of the shoulder correlate with the impact on physical function. This should be taken into account when planning an oncological resection, without compromising the oncological outcome.

In addition to the functional data, we were able to identify factors associated with a poor outcome such as a high-grade, local recurrence and (pulmonary) metastasis. Particularly, the high-grade tumors had a poor outcome in our cohort. This is in line with research in other studies [21]; our overall survival of 65% is lower than reported in other studies [5], which might be explained by the relatively high percentage of dedifferentiated and high-grade chondrosarcoma in our cohort. This might also explain the relative high tumor recurrence rate (25%) in our cohort: all recurrences occurred in the higher-grade tumors and none of the grade 1 tumors recurred.

This report should be interpreted in the light of its limitations. First of all this is a single-center retrospective study with a limited amount of patients and might therefore not reflect other centers' experience. Thereby the physical outcome was assessed for only half of the patients, primarily due to the high mortality in patients suffering from a high-grade tumor in the other half of the cohort. We can only speculate if the reported physical function is the same for these patients. We believe, however, that there is a more important role for the specific affected area, as shown in our results by the significant differences between patients who have their glenoid and rotator cuff intact and those who do not. However, we should take into account that there is an additional (relative) heterogeneity in the functional consequences after surgery since some patients are treated with an addition functional reconstruction based on the surgeon's preference. Finally, the group of clavicles consists only of 2 patients due to the rarity of primary chondrosarcoma of the clavicle, and therefore, it is impossible to state this group reflects other patients with by tumors affected clavicles.

In conclusion, patients treated with oncological resection of the scapula and/or clavicle have a considerable good upper extremity function that was predicated on preservation of the glenoid and rotator cuff. The patient's overall survival correlates with the development of pulmonary metastasis, local recurrence and tumor grade.

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PART IV

Summary and Discussion

8

Chapter 8

Summary

PART I – INTRODUCTION AND CHONDROSARCOMA STATISTICS

Chapter 1.

General introduction and outline of this thesis

Chondrosarcoma is the second most common primary bone malignancy. The clinical behavior of these tumors is closely related to their histological grading. Especially high-grade conventional (grade 2 and 3) and dedifferentiated chondromasarcoma are aggressive tumors with a poor prognosis and a high tendency to metastasize. Due to this poor prognosis, there is a need for more effective (systemic) therapies. Until now surgical resection remains the primary treatment for chondrosarcoma since radiation- and chemotherapy have limited effect on both conventional and dedifferentiated chondrosarcoma. In this thesis we explored different immunotherapeutic strategies in chondrosarcoma and we investigated the impact of oncological resections of the shoulder since this is one of the most common locations for both conventional and dedifferentiated chondrosarcoma.

Chapter 2.

The Identification of Prognostic Factors and Survival Statistics of Conventional Central Chondrosarcoma.

The aim of this review was to characterize the survival characteristics of conventional and dedifferentiated chondrosarcoma and identify independent predictive factors for both types of chondrosarcoma. This systematic review included 13 studies containing a total of 1114 patients. We showed that the prognosis for histologically low-grade tumors is generally good. In contrary we displayed a poor prognosis for patients with high-grade conventional chondrosarcoma and dedifferentiated chondrosarcoma. The prognostic factors identified for poor overall survival in conventional chondrosarcoma are high-grade tumors and an axial/pelvic tumor location. In dedifferentiated chondrosarcoma we identified the percentage of dedifferentiated component as a predictive factor on the disease-free survival. This study points out that there is a need for studies investigating new treatment options influencing survival of patients suffering from chondrosarcoma.

PART II - IMMUNOTHERAPEUTIC LEADS IN CHONDROSARCOMA

Chapter 3.

High TIL, HLA, and Immune Checkpoint Expression in Conventional High-Grade and Dedifferentiated Chondrosarcoma and Poor Clinical Course of the Disease.

The purpose of this study was to characterize chondrosarcoma tumor infiltration by immune cells and in addition characterize the expression of immune relevant molecules. We analyzed a self-developed tissue microarray containing 52 conventional and 24 dedifferentiated chondrosarcoma. By immunohistochemical staining we analyzed the expression of parameters associated with tumor-antigen specific immune responses. We focused in this study on CD4+ and CD8+ tumor infiltrating lymphocytes (TILs) and the expression of HLA class I heavy chain, beta-2 microglobulin, HLA class II and immune checkpoint molecules, B7-H3 and PD-1/PD-L1. We then correlated these staining results with the histopathological characteristics and the clinical outcomes.

We identified the CD8+ TILs in respectively 21% of the conventional chondrosarcoma and 90% of the dedifferentiated chondrosarcoma. B7-H3 was expressed in 69% of the conventional and 96% of the dedifferentiated chondrosarcoma. The expression of PD-1 and PD-L1 were identified in respectively 53% and 33% in the dedifferentiated tumors tested. We found an association of PD-L1 expression with a shorter time to metastasis in dedifferentiated chondrosarcoma.

This study suggests that chondrosarcoma are immunogenic tumors as indicated by the tumor infiltrating lymphocytes. However the defects in HLA class I antigen and the expression of the checkpoint molecules B7-H3 and PD-1/PD-L1 indicate that chondrosarcoma tumor cells utilize escape mechanisms to avoid immune recognition and subsequent destruction. These results can contribute to the rational design of immunotherapeutic strategies for the treatment of chondrosarcoma. Especially therapies targeting B7-H3 and PD-1/PD-L1 may have effect on these malignancies since they may counteract the immunosuppressive environment induced by B7-H3 and PD-1/PD-L1.

However this study also shows that there is a need for immunotherapeutic approaches that function independently of HLA I expression since HLA class I antigen expression was decreased in 77-92% of the conventional chondrosarcoma.

Chapter 4.

Chondroitin Sulfate Proteoglycan 4 expression in Chondrosarcoma: a potential target for antibody-based immunotherapy.

In this study we focussed on Chondroitin sulfate proteoglycan 4 (CSPG4) as a target for antibody-based therapy treating chondrosarcoma. CSPG4 is a cell surface proteoglycan that is highly expressed across various malignancies and has restricted distribution in healthy tissues, making it an attractive target for antibody-based therapy. Subsequently, we explored CSPG4 specific chimeric antigen receptor (CAR) T cell therapy, a tumor killing mechanism that is independent of HLA I antigen expression or functionality.

Using our tissue microarray, we immunohistochemically stained primary conventional and dedifferentiated chondrosarcoma. We stained the samples from 76 patients using CSPG4 specific monoclonal antibodies. In addition we incubated 2 chondrosarcoma cell lines with CSPG4 specific CAR T cells to evaluate the efficacy of this therapy in lysing chondrosarcoma cells *in vitro.*

We identified medium to high expression of CSPG4 in 29 of 41 (71%) conventional chondrosarcoma tumors and in 3 of 20 (15%) dedifferentiated chondrosarcoma tumors. We showed a positive association between CSPG4 expression and time to metastasis in both subtypes. In the cell lines treated with CSPG4 CAR T there was a lysis of respectively >80% and 70% of the chondrosarcoma cells.

We show that CSPG4-targeted CAR T cells are effective in killing CSPG4-positive chondrosarcoma tumors and may be an effective adjuvant therapy in CSPG4-positive conventional and dedifferentiated chondrosarcoma tumors. This strategy might be of particular relevance since we showed that HLA class I expression is frequently reduced in conventional chondrosarcoma and this therapy functions independently of HLA class I expression.

PART III - OUTCOMES AFTER ONCOLOGICAL RESECTION AROUND THE SHOULDER

Chapter 5.

Outcome After Reconstruction of the Proximal Humerus for Tumor Resection: A Systematic Review.

In this this review we focused on determining the best way of reconstructing the proximal humerus after an oncological resection for malignant or aggressive benign bone tumors of the shoulder. The proximal humerus is a commonly affected location for chondrosarcoma and other tumors of the appendicular skeleton. By performing

a systematic review we tried to reveal which surgical reconstruction offers the best functional outcome, longest implant survival and lowest complication rate. The included reconstructions were endoprostheses, osteoarticular allografts and allograft prosthesis composites. Our systematic review included 29 studies containing 693 patients. Due to the substantial heterogeneity and bias we narratively reported our results.

In the prosthesis studies the functional scores ranged from 61% to 77% (10 studies, 141 patients), as measured by the Musculoskeletal Tumor Society (MSTS) score. In the osteoarticular allograft studies the functional outcomes ranged from 50% to 78% (8 studies, 84 patients) and in the allograft prosthesis composite studies the functional scores ranged from 57% to 91% (10 studies 141 patients).

The implant survival in the prosthesis studies ranged between 0.38 and 1.0 (341 patients). In the osteoarticular allograft studies the survival ranged from 0.33 to 1.0 (143 patients) and in the allograft prothesis composite group the survival ranged from 0.33 to 1.0 (132 patients).

The overall complication per patient varied between 0.045 and 0.85 in the prosthesis group. In the osteoarticular allografts studies the complication rate ranged between 0 en 1.5 and in the allograft prosthesis composite studies the complication rate ranged from 0.19 to 0.79 per patient. There was a higher fracture rate for the osteoarticular allografts but other specific complications were similar.

In this systematic review we found similar functional outcomes and survival rates between prosthesis and allograft protheses composites while seemingly avoiding fractures that are observed in osteoarticular allografts. In addition this study indicates the need for further collaboration in the field of surgical oncology using comparative trials to identify superiority of any particular treatment.

Chapter 6.

Functional Outcomes and Complications After Oncologic Reconstruction of the Proximal Humerus.

In line with previous review, we investigated the best method for an articular reconstruction after surgical resection of the proximal humerus. We selected a cohort of 150 patients in 2 hospitals who underwent a wide resection of the proximal humerus. This oncological resection was followed the 3 earlier mentioned oncologic reconstructions: endoprostheses, osteoarticular allografts and allograft-prosthesis composites.

We prospectively collected the data from 25 patients in this cohort, with the QuickDASH being our main outcome. This QuickDASH provides a disability score

ranging from zero to 100, with a higher score representing higher disability. The average Disabilities of the Arm, Shoulder and Hand (QuickDASH) questionnaire score was 26 in this group. In comparison, the average DASH score for the American population is 10 ± 15.

There were no differences observed between the 3 reconstructional methods as measured with the QuickDASH, the upper extremity Toronto Extremity Salvage Score, the upper extremity Musculoskeletal Tumor Society score, and the Patient-Reported Outcomes Measurement Information System (PROMIS) score.

Subsequently, we retrospectively assessed complications and implant survival. Fractures, component loosening and nonunion of the reconstruction were more common in the OA group compared to the other 2 groups. We found no differences in postoperative infection, subluxation and dislocation, proximal humerus migration, or nerve and wound complications among the different reconstruction techniques.

This study showed an overall implant survival of >50%, with revision surgery as an endpoint for our survival analysis. There was a higher failure rate in the osteoarticular group compared to the other 2 constructs.

The functional ranges reported in our functional cohort (n = 21; mean, 64%; range, 53% to 80%; IQR, 73% to 87%) are comparable to our previous review (chapter 5). The complication rates are also comparable regarding infection, subluxation, dislocation, proximal migration of the humerus, and delayed union and this large series conforms a higher fracture rate in osteoarticular allografts compared to the other 2 methods. This higher fracture rate explains the observed higher revision rate and apparent lower survival rate in this group compared to the endoprosthesis and allograft prosthesis composites.

Chapter 7.

Functional and Oncological Outcome of Surgical Resection of the Clavicle and Scapula for Primary Chondrosarcoma.

We focus in this study on the functional and oncological outcome of patients with a primary chondrosarcoma of the clavicle and scapula that were treated operatively. We selected a sample of 20 patients that received the diagnosis of a primary chondrosarcoma of the clavicle or scapula. In 18 patients the tumor was located in the scapula and 2 patients suffered from a chondrosarcoma of the clavicle. We assessed the patients' function using the DASH and the PROMIS Physical Function - Upper Extremity. The oncological outcome was registered by tracking patients longitudinally.

For the 18 patients suffering from a chondrosarcoma of the scapula, the average DASH score was 16 ± 16 and the average PROMIS Physical Function – Upper Extremity score was 48 ± 10. We observed a better physical function in patients with an intact rotator cuff and glenoid. Also there was an association between decreased overall survival and metastasis, local recurrence and a higher tumor grade.

Aims and Key findings

Aims of the thesis

Part I

- Characterize the survival characteristics for conventional and dedifferentiated chondrosarcoma.
- Identify independent predictive factors for conventional and dedifferentiated chondrosarcoma.

Part II

- Identify tumor infiltration lymphocytes in conventional and dedifferentiated chondrosarcoma.
- Specify defects in HLA class I antigen processing machinery in conventional and dedifferentiated chondrosarcoma.
- Investigate the presence of the immune checkpoint molecules B7-H3 and PD-1/PD-L1 in conventional and dedifferentiated chondrosarcoma.
- Assess the expression of CSPG4 in conventional and dedifferentiated chondrosarcoma.
- Determine the effectiveness of CSPG4-specific CAR T cell therapy in eliminating chondrosarcoma cells *in vitro*.

Part III

- Identify which treatment option has the best functional result comparing: endoprostheses, osteoarticular allografts and allograft-prosthesis composites.
- Determine which of these treatment options have the longest implant survival and the lowest complication rate.
- Characterize the functional and oncological outcome for patients treated operatively for scapular or clavicular chondrosarcoma.

Key findings

Part I

- In chondrosarcoma the prognosis for histologically low-grade tumors is generally good. In contrary we showed a poor prognosis for patients with high-grade conventional chondrosarcoma and dedifferentiated chondrosarcoma.
- High-grade tumors and an axial/pelvic tumor location are prognostic factors for poor overall survival in conventional chondrosarcoma
- In dedifferentiated chondrosarcoma the percentage of dedifferentiated component is a predictive factor on the disease-free survival.

Part II

- Chondrosarcoma are immunogenic tumors as indicated by the tumor infiltrating lymphocytes.
- Defects in HLA class I antigen and the expression of the checkpoint molecules B7-H3 and PD-1/PD-L1 indicate that chondrosarcoma tumor cells utilize escape mechanisms to avoid immune recognition and subsequent destruction.
- The results of this thesis contribute to the rational design of immunotherapeutic strategies for the treatment of chondrosarcoma, such as systemic therapies targeting B7-H3 and PD-1/PD-L1. This may have a positive effect on chondrosarcoma since they may counteract the immunosuppression induced by B7-H3 and the PD-1 axis and may also inhibit the metastatic potential.
- We show CSPG4, which is commonly expressed in chondrosarcoma, as a promoter of disease and therefore as a clinically relevant target in patients with chondrosarcoma
- We provide a rationale for future studies to investigate the effectiveness of CSPG4 directed therapy in chondrosarcoma, such a CSPG4-targeted CAR T cell immunotherapy, *in vivo*.

Part III

- Endoprostheses, osteoarticular allografts, and allograft-prosthesis composites seem largely comparable in functional outcome and implant survival where overall complications seems higher in the osteoarticular allograft group based on higher osteoarticular allograft fracture rates.
- Patients treated with oncological resection of the scapula and/or clavicle have a considerable good upper extremity function that was predicated on preservation of the glenoid and rotator cuff. The patient's overall survival correlates with the development of pulmonary metastasis, local recurrence and tumor grade.

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Chapter 9

General discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

As described in this thesis, chondrosarcoma are the second most common primary malignant bone tumors with a clinical behavior closely associated with the histological grade. Low-grade chondrosarcoma behaves indolently, but high-grade chondrosarcoma (grade 2 and 3) as well as dedifferentiated chondrosarcoma are aggressive tumors with a poor prognosis and high risk of metastasis. Due to their poor prognosis these high-grade chondrosarcoma and dedifferentiated chondrosarcoma are good candidates for exploring more effective systemic treatment options. Until now surgical resection remains the primary treatment since radiation- and chemotherapy have limited effect on both conventional and dedifferentiated chondrosarcoma. In this thesis we focused on potential immunotherapeutic leads giving direction to future systemic therapies.

In addition, being one of the most common locations for both conventional and dedifferentiated chondrosarcoma, we evaluated the impact of oncological resections of chondrosarcoma around the shoulder. Comparing endoprostheses, osteoarticular allografts and allograft-prosthesis composites, we evaluated the best functional result as well as implant survival and complication rate. Finally, we characterized the functional and oncological outcome for patients treated operatively for scapular or clavicular chondrosarcoma.

Immunotherapeutic leads in chondrosarcoma

In this thesis we analyzed if patients with chondrosarcoma develop a T cell immune response against their own tumor and we analyzed the expression of immunologically relevant molecules on chondrosarcoma cells, such as HLA class I subunits, HLA class II antigens and the immune checkpoints B7-H3 and PD-L1. This information will help us to better understand the role of immune surveillance in chondrosarcoma.

We showed that low-grade conventional chondrosarcoma is less immunogenic than high-grade conventional chondrosarcoma as indicated by limited TILs and lower HLA class I and HLA class II expression. Moreover, dedifferentiated chondrosarcoma is more immunogenic than conventional chondrosarcoma as indicated by comparing the same markers.

As observed in most other solid cancers, HLA class I defects were found in chondrosarcoma. HLA class I antigen expression was decreased in 77-92% of the conventional chondrosarcoma compared to only 0-15% of the dedifferentiated chondrosarcoma. This shows that the frequency of defects is significantly lower in subtypes with an aggressive phenotype (high-grade and dedifferentiated chondrosarcoma) compared to those with a benign phenotype and consequent favourable clinical course of the disease. This is an unexpected finding since in

general a defective HLA class I antigen expression is associated with a poor clinical course of the disease (1). Several mechanisms might possibly explain this unexpected association between a defective HLA class I antigen expression and a favourable clinical course of the disease.

One possibility is that immunosurveillance does not play a role in the pathogenesis of chondrosarcoma. This mechanism seems unlikely since we found TILs indicating the hosts recognizing and developing a T cell mediated immune response to tumor antigens expressed by their own tumors.

Another explanation would be that NK cells instead of T cells play the major role in the elimination of malignant cell as this mechanism has been described in other cancers (2, 3). Hereby a high HLA class I antigen expression would serve as a defensive mechanism for tumor cells since it would inhibit the ability of NK cells to eliminate these cells.

The last possibility, that we believe is most likely, is that patients mount an immune response to the tumor antigens expressed by their own tumors, however this response does not lead to antitumor activity because it is inhibited by an immunosuppressive environment. Immune checkpoint molecules can facilitate such an immunosuppressive environment. Therefore we next analyzed 2 relevant immune checkpoint molecules, B7-H3 and PD-L1.

B7-H3 was expressed in 96% of the dedifferentiated chondrosarcoma and in 69% of the conventional chondrosarcoma. B7-H3 has been shown to have an inhibitory immune effect by preventing activation of CD8+ T lymphocytes (4). In many other solid cancers B7-H3 has already been shown to inhibit the antitumor activity of T cells and B7-H3 has been associated with immune suppression and inferior prognosis (5, 6). B7-H3 is an attractive target for immunotherapy due to the preferred expression on tumor cells and limited expression on normal tissue (7, 8).

PD-L1 was expressed in 33% of the dedifferentiated chondrosarcoma and we found an association between PD-L1 expression on chondrosarcoma cells and time to metastasis. The number of tumor samples is small, so this is important to take in mind when interpreting these results. However, in other cancers PD-L1 expression has also been associated with metastatic disease (9, 10). We didn't find any expression of PD-L1 in conventional chondrosarcoma.

Therefore, therapies targeting B7-H3 and PD-1/PD-L1 may have a beneficial effect on B7-H3 and/or PD-L1 positive chondrosarcoma since they may counteract the immunosuppression induced by B7-H3 and the PD-1 axis and may also inhibit the metastatic potential.

Currently several clinical trials are ongoing to investigate the effect of immunotherapy, such as anti-PD-1, on chondrosarcoma and other sarcoma (11). Paoluzzi et al. report a partial response of a patient with dedifferentiated chondrosarcoma with nivolumab (an anti-PD1 monoclonal antibody) (12).

To investigate potential immunological therapies for patients with chondrosarcoma with defective HLA class I expression we focused on chimeric antigen receptor (CAR) T cell therapy. This treatment option is independent of HLA I antigen expression. Since HLA class I expression is frequently decreased in conventional chondrosarcoma, this CAR T cell therapy is potentially of particular relevance for these patients.

CAR T cell therapy is a strategy where T cells are genetically engineered to express tumor specific antibody fragments to target and kill cancer cells (13). This therapy has been effective in leukemia but has been less effective in solid tumors due to shared antigens between tumor and normal tissue, poor tumor core infiltration and low density of tumor specific antigens.

We choose cell surface proteoglycan CSPG4 as target, which is highly expressed across various cancers. Its restricted expression on healthy tissue makes CSPG4 a suitable target for antibody-based immunotherapy. We showed that medium to high CSPG4 expression is associated with shorter time to metastasis and decreased overall survival in conventional and dedifferentiated chondrosarcoma, which is in line with findings in other malignancies (14, 15). This finding identified CSPG4 as a promotor of disease and is therefore a relevant target in patients with chondrosarcoma.

In other cancers genetically engineered CSPG4-targeted CAR T cells have proven to control tumor growth *in vitro* and *in vivo* in with different cell lines engrafted NSG mice (human melanoma, head and neck squamous cell carcinoma and breastcarcinoma). Also, CSPG4-targeted CAR T cells have demonstrated to lyse CSPG4 expressing glioblastoma cancer stem cells (16, 17). In this thesis we observed the killing of CSPG4 chondrosacoma cells *in vitro* when incubated with CSPG4-targeted CAR T cells. This indicates that CSPG4-targeted CAR T cell immunotherapy is as a potential effective adjuvant therapy in CSPG4-positive conventional and dedifferentiated chondrosarcoma.

This thesis should be interpreted with its limitations in mind. Caution has to be exercised in interpreting our findings since the number of tumor samples analysed is small. Our results should be independently confirmed by analysing a large number of samples to determine whether our data is generalizable.

The results of this thesis contribute to the rational design of immunotherapeutic strategies for the treatment of chondrosarcoma, in particular because immunosurveillance appears to play a role in the clinical course of the disease. Better

and more evidenced based treatments might prevent excessive exposure of patients to potentially harmful therapies such as radiation- and chemotherapy.

Outcomes after oncological resection around the shoulder

Chondrosarcoma are commonly located around the shoulder region. The oncological resection and subsequent surgical reconstruction potentially affects shoulder function and may cause complications. There is no consensus yet about the best reconstructive methods after oncological resection of the proximal humerus. In this thesis we compared endoprostheses, osteoarticular allografts, and allograft-prosthesis composites. We performed a systematic review and subsequently collected and analyzed prospective functional data of 2 institutions, identifying which of these 3 surgical methods offers the best functional outcome, has the longest survival rate and lowest complication rate after proximal humerus resection.

Our review showed the majority of the MSTS score for the 3 constructs were between 60% and 79%, making them largely comparable in functional outcome. These results are comparable with the results observed in our functional prospective cohort (n = 21; mean, 64%; range, 53% to 80%).

Also implant survival looks similar between the 3 constructs as observed in our review and functional cohort. The overall complications per patient seem greater in the osteoarticular group compared to the other 2. This observed higher complication rate seems to be based on a higher fracture rate in the osteoarticular allograft group. Allograft-prosthesis composites and prostheses are comparable based on the literature, and both seem to avoid the problem of fracture that is observed with osteoarticular allografts.

For future research, multicenter studies are needed if we want to establish the optimal treatment for oncological resections around the shoulder and a prospective database including patients from specialized treatment centers would be an essential first step.

Finally, we assessed the functional and oncological outcome of chondrosarcoma of the clavicle and scapula. We report the prospectively data of 10 patients and observed a generally good function. We observed an average DASH score of 16, and the mean PROMIS Physical Function-Upper Extremity score was 48 for concering the patients with a (partial) scapulectomy. Remarkably, the observed function in our cohort is comparable to that of the reported disability of the general population (average DASH is 10 in the American population (18)).

When the rotator cuff and glenoid were still intact a better physical function was observed than in patients with those structures affected. This is in line with earlier research indicating that the size of the resection and the involved anatomical

structures in these parts of the shoulder correlate with the impact on physical function (19). This should be taken into account when performing an oncological resection, without compromising adequate resection. In addition, we were able to determine factors associated with a poor oncological outcome such as a high-grade tumors, local recurrence and (pulmonary) metastasis. In this cohort particular the high-grade chondrosarcoma had a poor outcome which is in line with other studies (20).

When interpreting the results of this thesis, its limitation should be taken into account. Our data has been gathered in specialized academic hospitals representing the experience of a small number of surgeons with a limited amount of patients. Therefore these results may not reflect the results of others. In addition, the studies in our review as well as our own functional prospective cohort are nonrandomized, thereby increasing the risk of (selection) bias. Further, there are restrictions in the comparability of the 3 reconstruction techniques which are partially caused by the deviations in the indications for surgery. The relatively different indications and treatments between primary and metastatic disease are likely to have influenced the comparability the most. The limited number of patients in our analyzed studies does not allow for extensive subgroup analysis to evaluate different clinical scenarios such a tumor type and soft tissue resection. This affects the predictive value for specific patients.

The continuous progress in the systemic treatment for different cancers and sarcoma will presumably lead to smaller oncological resections and subsequent better functional outcomes. In the future effective therapy might even make the need for surgical resections abundant for specific patients hereby preventing surgical risks and limiting the functional impact.

For future research we suggest reporting and gathering functional data from a patient-rated perspective instead of physician-rated outcomes to prevent an overestimation of the function, while facilitating the possibility of combining raw data to elucidate differences between treatment options. Finally, we encourage further centralization of care for patients with relatively rare diseases, which might give opportunities for researchers to set up prospective and comparative studies. Hereby including large groups of patients using standardized surgeries, perioperative care and outcome reporting.

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APPENDICES

APPENDICES BY CHAPTER

Appendix chapter 2

Appendix

Author, year	**Study participation**	**Study attrition**	**Confounding measurement**
	Dates of researched period stated. Clearly defined patient sample, assembled at a common point in course of the disease.	Sufficiently long and complete follow up (≥2 years and ≥80%). Explaining reasons for patients being lost to follow up	Defined and comparable treatment for patients
Andreou, 2011	1	1	1, types of surgery mentioned
Angelini, 2012	1	1	1, types of surgery mentioned
Briccoli, 2002	1	0	1, types of surgery mentioned
Cho, 2011	1	1	1, types of surgery mentioned
De Camargo, 2010	1	1	1, types of surgery mentioned
Donati, 2010	1	1	1, types of surgery mentioned
Donati, 2005	1	1	1, types of surgery mentioned
Gitelis, 1981	1	1	1, types of surgery mentioned
Mavrogenis, 2013	1	1	1, types of surgery mentioned
Mitchell, 2000	1	0	1, types of surgery mentioned
Ozaki, 1996 (2, Cancer)	1	1	1, types of surgery mentioned
Staals, 2006	1	0	1, types of surgery mentioned
Van Maldegem et al, 2014	1	0	1, types of surgery mentioned

Author, year	**Analysis**	**Population**	**Disclosure**
	Valid statistical analysis is done. Multivariable analysis is done	(no overlap)	
Andreou, 2011	1	1	1
Angelini, 2012	1	0	1
Briccoli, 2002	0	0	0
Cho, 2011	0	1	1
De Camargo, 2010	0	1	1
Donati, 2010	0	0	1
Donati, 2005	1, but not on survival central	0	1
Gitelis, 1981	0	0	0
Mavrogenis, 2013	1	0	1
Mitchell, 2000	0	1	1
Ozaki, 1996 (2, Cancer)	0	1	0
Staals, 2006	0	0	0
Van Maldegem et al, 2014	0	0	1

Author, year	**Prognostic factor measurement**	**Outcome measurement**
	Clear definition and valid assessment of prognostic factors	Well defined outcome parameters (Survival: overall, metastasic free, event free)
Andreou, 2011	1	1
Angelini, 2012	1	1
Briccoli, 2002	0	0
Cho, 2011	1	1
De Camargo, 2010	1	1
Donati, 2010	1	0
Donati, 2005	1	0
Gitelis, 1981	1	1
Mavrogenis, 2013	1	1
Mitchell, 2000	1	0
Ozaki, 1996 (2, Cancer)	1	1

Staals, 2006	1	1
Van Maldegem et al, 2014	1	1

Author, year	**FU**	**Level of evidence**
	≥ 1year	I-IV
Andreou, 2011	1	4, prognostic
Angelini, 2012	1	4, prognostic
Briccoli, 2002	0	4, prognostic
Cho, 2011	1	3, therapeutic
De Camargo, 2010	1	4, prognostic
Donati, 2010	1	*2, prognostic*
Donati, 2005	1	4, prognostic
Gitelis, 1981	1	4, prognostic
Mavrogenis, 2013	1	4, prognostic
Mitchell, 2000	1	4, prognostic
Ozaki, 1996 (2, Cancer)	1	*4, prognostic*
Staals, 2006	0	4, prognostic
Van Maldegem et al, 2014	1	4, prognostic

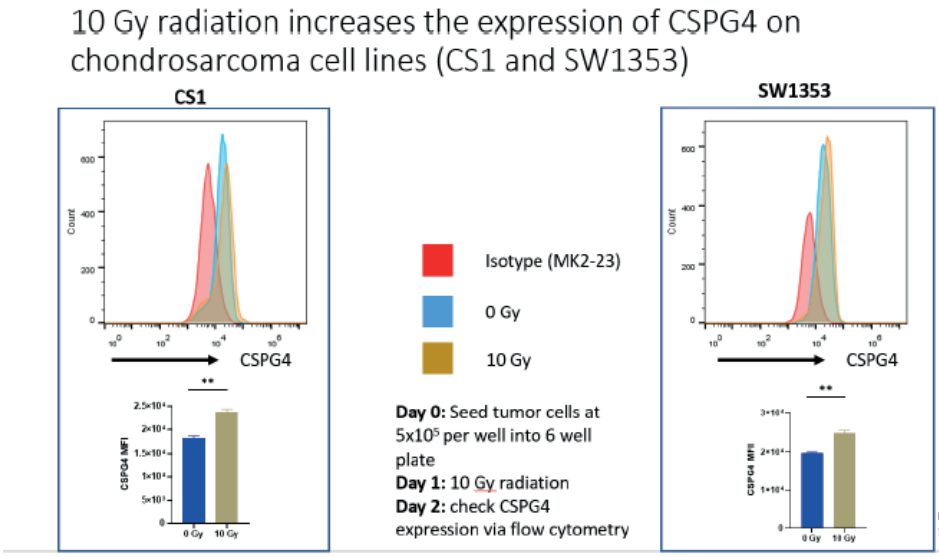
Author, year	**Confounding measurement**	**Baseline**
	Defined and comparable treatment for patients	
Andreou, 2011	1, types of surgery mentioned	0
Angelini, 2012	1, types of surgery mentioned	0
Briccoli, 2002	1, types of surgery mentioned	1
Cho, 2011	1, types of surgery mentioned	0
De Camargo, 2010	1, types of surgery mentioned	0
Donati, 2010	1, types of surgery mentioned	1

Donati, 2005	1, types of surgery mentioned	0
Gitelis, 1981	1, types of surgery mentioned	1
Mavrogenis, 2013	1, types of surgery mentioned	0
Mitchell, 2000	1, types of surgery mentioned	1
Ozaki, 1996 (2, Cancer)	1, types of surgery mentioned	1
Staals, 2006	1, types of surgery mentioned	0
Van Maldegem et al, 2014	1, types of surgery mentioned	1

Author, year	**Disclosure**
Andreou, 2011	1
Angelini, 2012	1
Briccoli, 2002	0
Cho, 2011	1
De Camargo, 2010	1
Donati, 2010	1
Donati, 2005	1
Gitelis, 1981	0
Mavrogenis, 2013	1
Mitchell, 2000	1
Ozaki, 1996 (2, Cancer)	0
Staals, 2006	0
Van Maldegem et al, 2014	1

Appendix chapter 4

Supplementary Figure 1. CSPG4 expression in CS1 and SW1353 chondrosarcoma cells increases with with one subclinical dose of irradiation (10 Gy).



Appendix chapter 5

Appendix 1. Critical appraisal

Study	Year	Level of evidence	Disclosure	Patient selection	Complication reporting	Outcome assessment	Baseline	Postoperative rehabilitation
Burrows et al. [11]	1975	IV	-	+	-	-	+	-
Bos et al. [10]	1987	IV	-	-	-	-	+	-
Gebhardt et al. [23]	1990	IV	NA	NA	-	-	+	-
Aho et al. [3]	1994	IV	-	+	-	-	-	-
Alman et al. [4]	1995	IV	+	+	-	-	-	-
Jensen & Johnston [30]	1995	IV	-	+	-	-	+	+
Malawer et al. [35]	1995	IV	+	-	+	-	-	-
O'Connor et al. [44]	1996	III	+	+	-	-	-	-
Meller et al. [41]	1997	IV	-	-	-	-	+	-
Probyn et al. [46]	1998	III	-	-	-	-	+	+
Getty & Peabody [24]	1999	IV	+	-	-	-	+	-
Voggenreiter et al. [57]	1999	IV	-	+	+	-	+	-
Fuhrmann et al. [22]	2000	IV	-	-	-	-	+	+
Suk et al. [52]	2002	IV	-	-	-	-	+	-
Wittig et al. [61]	2002	IV	-	+	-	-	+	-
Kumar et al. [33]	2003	IV	-	-	-	+	+	+

Study	Year	Level of evidence	Disclosure	Patient selection	Complication reporting	Outcome assessment	Baseline	Postoperative rehabilitation
DeGroot et al. [15]	2004	IV	-	+	+	-	+	+
Mayilvahanan et al. [39]	2006	IV	-	-	-	-	+	-
Black et al. [9]	2007	IV	-	+	-	-	+	-
Kitagawa et al. [32]	2007	III	-	-	-	-	+	-
Sharma et al. [50]	2007	IV	+	+	-	-	-	-
Potter et al. [45]	2009	III	+	+	-	-	+	-
Abdeen et al. [1]	2009	IV	+	+	-	-	+	+
Ioannou et al. [29]	2009	IV	-	-	-	-	+	-
Moran & Stalley [42]	2009	IV	-	+	-	-	+	+
Manfrini et al. [36]	2011	III	+	+	-	-	-	+
van de Sande et al. [56]	2011	III	-	+	-	-	-	+
Wang et al. [60]	2011	III	+	-	-	-	-	-
Aponte-Tinao et al. [6]	2013	IV	+	+	+	-	-	-

	Disclosure		Outcome assessment
+	Reported	+	Outcome recording by independent assessor
-	Unreported	-	Outcome recorded by researcher or unspecified

	Patient selection		**Baseline**
+	Eligibility criteria, sources and methods of selection of participants described	+	Detailed baseline characteristics of proximal humerus construct
-	Potential selection bias/not described	-	Mixed or unspecified baseline characteristics
	Complication recording		**Postoperative rehabilitation**
+	Stated which complications to be recorded	+	Well described
-	Complications to be recorded not specified	-	Protocol not reported

Level of evidence according to Clinical Orthopaedics and Related Research guidelines; NA = not assessable

Appendix 2. Other complications

Study	Construct	Sample size	Infection	Proportion	Subluxation	Proportion	Dislocation	Proportion	Proximal migration	Proportion	Loosening	Proportion	Non-union	Proportion	Nerve Praxia	Proportion
Burrows et al. [11]	Prosthesis	6	0	0	1	0.17	0	0	0	0	3	0.50	0	0	0	0
Bos et al. [10]	Prosthesis	13	0	0	3	0.23	4	0.31	0	0	2	0.15	0	0	0	0
Malawer et al. [35]	Prosthesis	29	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
O'Conner et al. [44]	Prosthesis	11	1	0.09	6	0.55	0	0	0	0	1	0.09	0	0	0	0
Meller et al. [41]	Prosthesis	8	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Voggenreiter et al. [57]	Prosthesis	19	1	0.05	0	0	0	0	0	0	0	0	0	0	1	0.05
Fuhrmann et al. [22]	Prosthesis	22	1	0.05	0	0	0	0	0	0	0	0	0	0	0	0
Wittig et al. [61]	Prosthesis	15	0	0	0	0	0	0	0	0	1	0.07	0	0	0	0
Kumar et al. [33]	Prosthesis	45	1	0.02	0	0	2	0.044	0	0	6	0.13	0	0	1	0.02
Mayilvahanan et al. [39]	Prosthesis	55	2	0.04	0	0	1	0.018	6	0.11	2	0.04	0	0	2	0.04
Kitagawa et al. [32]	Prosthesis	5	1	0.20	2	0.40	0	0	0	0	0	0	0	0	0	0
Sharma et al. [50]	Prosthesis	21	0	0	3	0.14	0	0	0	0	0	0	0	0	0	0
Potter et al. [45]	Prosthesis	16	0	0	2	0.13	3	0.19	0	0	0	0	0	0	0	0
Ioannou et al. [29]	Prosthesis	12	1	0.08	0	0	1	0.083	0	0	0	0	0	0	0	0
Manfrini et al. [36]	Prosthesis	25	3	0.12	9	0.36	0	0	0	0	3	0.12	0	0	0	0
van de Sande et al. [56]	Prosthesis	14	0	0	0	0	1	0.071	6	0.43	0	0	0	0	0	0
Wang et al. [60]	Prosthesis	25	2	0.08	13	0.52	7	0.28	0	0	0	0	0	0	0	0

Study	Construct	Sample size	Infection	Proportion	Subluxation	Proportion	Dislocation	Proportion	Proximal migration	Proportion	Loosening	Proportion	Non-union	Proportion	Nerve Praxia	Proportion
Gebhardt et al. [23]	Allograft	20	3	0.15	1	0.05	0	0	0	0	0	0	1	0.050	0	0
Aho et al. [3]	Allograft	4	1	0.25	1	0.25	1	0.25	0	0	0	0	0	0	0	0
Alman et al. [4]	Allograft	3	0	0	1	0.33	0	0	0	0	0	0	0	0	0	0
O'Conner et al. [44]	Allograft	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Probyn et al. [46]	Allograft	10	2	0.20	3	0.30	1	0.10	0	0	0	0	0	0	0	0
Getty & Peabody [24]	Allograft	13	1	0.08	3	0.23	8	0.62	0	0	0	0	0	0	0	0
DeGroot et al. [15]	Allograft	31	1	0.03	2	0.07	1	0.032	0	0	0	0	0	0	1	0.03
Potter et al. [45]	Allograft	17	1	0.06	3	0.18	0	0	0	0	0	0	1	0.06	0	0
Manfrini et al. [36]	Allograft	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
van de Sande et al. [56]	Allograft	13	1	0.08	1	0.08	0	0	6	0.46	0	0	2	0.15	0	0
Aponte-Tinao et al. [6]	Allograft	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Jensen & Johnston [30]	APC	14	0	0	2	0.14	1	0.071	0	0	0	0	0	0	0	0
Suk et al. [52]	APC	6	0	0	0	0	0	0	0	0	0	0	1	0.17	0	0
Black et al. [9]	APC	6	0	0	0	0	0	0	0	0	1	0.17	1	0.17	0	0
Potter et al. [45]	APC	16	2	0.13	3	0.19	0	0	0	0	0	0	1	0.06	0	0
Abdeen et al. [1]	APC	36	0	0	0	0	1	0.028	5	0.14	3	0.08	0	0	0	0
Moran & Stalley [42]	APC	11	0	0	4	0.36	0	0	0	0	0	0	2	0.18	0	0
Manfrini et al. [36]	APC	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Study	Construct	Sample size	Infection	Proportion	Subluxation	Proportion	Dislocation	Proportion	Proximal migration	Proportion	Loosening	Proportion	Nonunion	Proportion	Nerve Praxia	Proportion
van de Sande et al. [56]	APC	10	1	0.1	3	0.30	1	0.1	8	0.8	0	0	0	0	0	0
Wang et al. [60]	APC	14	1	0.07	4	0.29	3	0.21	0	0	0	0	3	0.21	0	0
Aponte-Tinao et al. [6]	APC	16	0	0	0	0	0	0	0	0	0	0	2	0.13	0	0

MSTS = Musculoskeletal Tumor Society, APC = allograft-prosthesis composite, NR = not reported

Appendix chapter 6

Table 1: Demographics		**n=150**
Sex	**n**	**%**
male	74	49
female	76	51
Location procedure		
right arm	73	49
Left arm	77	51
Type of reconstruction		
osteoarticular allograft	46	31
prosthesis	84	56
allograft prosthesis composite	20	13
Operated on shoulder before reconstruction		
yes	22	15
no	128	85
Institution		
hospital 1	111	74
hospital 2	39	26
Primary vs. metastatic disease		
primary	61	41
metastatic	89	59

	median (IQR)	**range**
Age at time of diagnosis*	55 (42-65)	9.2 - 88
Age at time of reconstruction	56 (42-65)	9.4 - 93
Follow-up time, years	2.3 (0.42-6.5)	0.0082-40

	mean ± SD	**range**
Age at time of diagnosis*	52 ± 19	9.2 - 88
Age at time of reconstruction	53 ± 19	9.4 - 93
Follow-up time, years	5.0 ± 6.6	0.0082-40

*n=144

Table 2: Operation statistics **n=150**

	Osteoarticular Allograft	%	Prosthesis	%	Allograft Prosthesis Composite	%	P-value
Sex							
male	24	52	39	46	11	55	0.76
female	22	48	45	54	9	45	
Operated on shoulder before reconstruction							
yes	9	20	10	12	3	15	0.49
no	37	80	74	88	17	85	
Pathologic fracture							
yes	29	63	14	17	10	50	<0.001
no	17	37	70	83	10	50	
Right sided procedure							
yes	23	50	42	50	8	40	0.74
no	23	50	42	50	12	60	
Metastatic disease							
yes	15	**33**	64	**76**	10	**50**	<0.001
no	31	67	20	24	10	50	
Glenoid removed							
yes	11	**24**	3	**4**	0	0	<0.001
no	35	76	81	96	20	100	
Scapula removed							
no	41	89	80	95	20	100	0.15
1/3 of scapula	5	11	2	2.4	0	0	
2/3 of scapula	0	0	2	2.4	0	0	
total	0	0	0	0	0	0	

Clavicle removed							
no	44	94	80	95	20	100	1.0
1/3 of clavicle	1	2	3	3.6	0	0	
2/3 of clavicle	1	2	1	1.2	0	0	
total	0	0	0	0	0	0	
Deltoid removed							
no	26	**57**	73	**87**	14	**70**	0.001
1/3 of deltoid	8	17	8	10	5	25	
2/3 of deltoid	8	17	2	2.4	1	5.0	
total	4	**8.7**	1	**1.2**	0	0	
Spica cask revalidation							
yes	17	**37**	3	3.6	5	**25**	<0.001
no	29	63	81	96	15	75	
Malawer classification							
1a	24	52	71	85	14	70	
1b	11	24	10	12	6	30	
5a	2	4	2	2	0	0	<0.001
5b	9	20	1	1	0	0	
Rotator cuff intact							
yes	37	80	74	88	18	90	0.43
no	9	20	10	12	2	10	
Operated on shoulder after reconstruction (including revisions)							
yes	21	**46**	14	**17**	4	**20**	0.001
no	25	54	70	83	16	80	
Revision surgery on shoulder performed							
yes	19	**41**	10	**12**	3	**15**	<0.001
no	27	59	74	88	17	85	

	median (IQR)	range	median (IQR)	range	median (IQR)	range	P-value
Size resection, cm	14 (11-16)	5.5-26	11 (9-13)	0-30	14 (11-18)	7-23	<0.001
Total number of reoperations	0 (0-2)	0-6	0 (0-0)	0-5	0 (0-0)	0-2	0.025
Age at time of reconstruction	36 (25-56)	9-77	61 (53-70)	25-93	57 (37-64)	15-77	<0.001
Follow-up time, years	7.3 (1.9-15)	0.0082-40	1.1 (0.24-4.0)	0.0082-23	3.8 (1.2-6.5)	0.19-14	0.001
	mean ± SD,	**range**	**mean ± SD, range**	**range**	**mean ± SD, range**	**range**	**P-value**
Size resection, cm	14 ± 3.9	5.5-26	11 ± 5.4	0-30	14 ± 4.5	7-23	0.0035
Total number of reoperations	1.2 ± 1.7	0-6	0.44 ± 1.3	0-5	0.25 ± 0.55	0-2	0.0070
Age at time of reconstruction	40 ± 20	9-77	61 ± 13	25-93	49 ± 19	15-77	<0.001
Follow-up time, years	9.6 ± 8.8	0.0082-40	2.6 ± 3.7	0.0082-23	4.6 ± 4.1	0.19-14	<0.001

Table 3: Complication statistics							n=150
	Osteoarticular Allograft	%	Prosthesis	%	Allograft Prosthesis Composite	%	P-value
Infection							
yes	5	**11**	9	**11**	2	**10**	1.0
no	41	89	75	89	18	90	
Fracture							
yes	22	**49**	4	**4.8**	2	**10**	<0.001
no	23	51	80	95	18	90	
Subluxation							
yes	14	**30**	37	**44**	9	**45**	0.27
no	32	70	47	56	11	55	
Dislocation							
yes	5	**11**	8	**10**	2	**10**	0.93
no	41	89	76	90	18	90	
Proximal migration							
yes	3	**6.5**	15	**18**	1	**5.0**	0.13
no	43	93	69	82	19	95	
Component/Hardware loosening							
yes	5	**11**	1	**1.2**	0	0	0.032
no	41	89	83	99	20	100	
Nonunion							
yes	5	**11**	0	0	1	**5.0**	0.007
no	41	89	84	100	19	95	
Malunion							
yes	0	**0**	0	**0**	0	**0**	N/A
no	46	100	84	100	20	100	
Nerve complications							
yes	2	**4.3**	5	**6.0**	2	**10**	0.64
no	44	96	79	94	18	90	
Wound complications							
yes	2	**4.3**	8	**9.5**	2	**10**	0.62
no	44	96	76	90	18	90	

Table 4: Survival analysis, excluding revisions for tumor recurrence/progression

	Events observed	Events expected	P-value
Type of reconstruction			
osteoarticular allograft	17	11	0.070
prosthesis	6	10	
allograft prosthesis composite	2	4	
Sex			
male	13	12	0.83
female	12	13	
Location procedure			
Right arm	14	13	0.57
Left arm	11	12	
Operated on shoulder before reconstruction			
yes	6	4.3	0.36
no	19	21	
Institution			
hospital 1	20	21	0.67
hospital 2	5	4.2	
Primary vs. metastatic disease			
primary	18	16	0.36
metastatic	7	9.2	
Pathologic fracture			
yes	10	12	0.51
no	15	13	
Glenoid removed			
yes	3	2.3	0.60
no	22	23	
Scapula removed			
no	25	23	0.42
1/3 of scapula	0	0.98	
2/3 of scapula	0	0.62	
Clavicle removed			
no	25	23	0.43
1/3 of clavicle	0	1.3	
2/3 of clavicle	0	0.23	
Deltoid removed			

	Events observed	Events expected	P-value
no	13	18	0.12
1/3 of deltoid	8	4.9	
2/3 of deltoid	4	2.0	
total	0	0.45	
Deltoid (partially) removed			
no	12	7.3	0.039
yes	13	18	
Spica cask revalidation			
yes	7	5.6	0.51
no	18	19	
Malawer classification			
1a	13	17	0.15
1b	9	6.0	
5a	0	0.91	
5b	3	1.4	
Rotator cuff intact			
yes	22	22	0.88
no	3	2.8	
Operated on shoulder after reconstruction (if applicable before revision)			
yes	8	3.7	0.015
no	17	21	
Infection			
yes	10	2.6	<0.001
no	15	22	
Fracture			
yes	17	6.8	<0.001
no	8	18	
Subluxation			
yes	9	12	0.31
no	16	13	
Dislocation			
yes	8	3.8	0.017
no	17	21	
Proximal migration			
yes	4	4.1	0.94
no	21	21	

	Events observed	Events expected	P-value
Component loosening			
yes	4	1.4	0.025
no	21	24	
Nonunion			
yes	3	1.4	0.16
no	22	24	
Nerve complications			
yes	4	1.4	0.024
no	21	24	
Wound complications			
yes	5	1.3	<0.001
no	20	24	
	Haz. Ratio	**SE**	**P-value**
Age at time of reconstruction	0.97	0.010	0.004
Size resection, cm	1.0	0.040	0.90

Table 5: Functional outcomes

Questionnaire	n	mean ± SD	median (IQR)	range
QuickDASH	25	26 ± 16	23 (18-41)	0-59
PROMIS UE	22	38 ± 6.0	38 (34-42)	25-49
MSTS	21	64 ± 20	67 (73-87)	53-80
TESS	22	81 ± 13	82 (72-90)	47-99
	n	**Median (IQR)**	**P-value**	
Reconstruction				
Osteoarticular Allograft	8	26 (22-35)	0.70	
Prosthesis	11	20 (14-45)		
Allograft Prosthesis Composite	6	19 (11-30)		
Sex				
male	10	20 (18-27)	0.25	
female	15	25 (14-45)		
Operated on shoulder before reconstruction				
yes	4	26 (22-38)	0.25	
no	21	20 (14-41)		
Pathologic fracture				
yes	4	17 (8.0-31)	0.28	
no	21	23 (20-41)		
Right sided procedure				
yes	8	23 (17-35)	0.93	
no	17	23 (18-41)		
Metastatic disease				
yes	5	14 (4.5-20)	0.094	
no	20	24 (20-43)		
Glenoid removed				
yes	1	20 (NA)	0.68	
no	24	23 (16-41)		
Scapula removed				
no	24	23 (16-41)	0.68	
1rd	0	NA		
2rd	1	20 (NA)		
total	0	NA		
Clavicle removed				
no	23	23 (14-41)	0.83	
1rd	1	20 (NA)		
2rd	1	20 (NA)		
total	0	NA		

Deltoid removed			
no	19	20 (14-30)	0.26
1rd	5	41 (30-48)	
2rd	0	NA	
total	1	20 (NA)	
Deltoid (partially) removed			
no	19	20 (14-30)	0.18
yes	6	35 (20-48)	
Malawer classification			
1a	18	22 (14-31)	0.40
1b	6	35 (20-48)	
5a	1	20 (NA)	
5b	0	NA	
Spica cask revalidation			
yes	8	28 (22-35)	0.21
no	17	20 (11-41)	
Rotator cuff intact			
yes	22	23 (18-41)	0.64
no	3	20 (14-30)	
Operated on shoulder after reconstruction			
yes	10	25 (20-41)	0.39
no	15	20 (11-41)	
Revision surgery on shoulder performed			
yes	8	25 (20-38)	0.48
no	17	20 (14-41)	

Correlation of function between variables and other continuous variables

	QuickDASH		**PROMIS UE**	
Variable	**rho**	**P-value**	**rho**	**P-value**
QuickDASH	NA	NA	-0.64	0.0014
PROMIS UE	-0.64	0.0014	NA	NA
MSTS	-0.70	<0.001	0.56	0.0084
TESS	-0.67	<0.001	0.70	<0.001
Age at time of reconstruction	-0.38	0.060	-0.08	0.73
Size resection	0.056	0.80	-0.10	0.67
Number of reoperations	0.091	0.67	-0.20	0.36

Table 6: Diagnoses		**n=150**
	n	%
Metastatic disease	**89**	**59**
metastatic renal cell carcinoma	29	19
metastatic breast carcinoma	15	10
metastatic lung carcinoma	13	8.7
metastatic thyroid carcinoma	5	3.3
metastatic leiomyosarcoma (pelvis, 2x retroperitoneum)	3	2.0
metastatic chondrosarcoma	3	2.0
metastatic osteosarcoma	2	1.3
other metastatic disease (sarcoma)	6	4.0
other metastatic disease (non-sarcoma)	13	8.7
Primary disease	**61**	41
osteosarcoma	21	14
chondrosarcoma	18	12
Ewing sarcoma	4	2.7
multiple myeloma	2	1.3
plasmacytoma	2	1.3
liposarcoma	2	1.3
enchondroma	2	1.3
other primary disease	10	6.7

Appendix: Comparing the functional cohort with the rest of the cohort

	Funtional cohort		Rest of the cohort		
	n	**%**	**n**	**%**	**P-value**
Sex					
Male	10	40	64	51	0.38
Female	15	60	61	49	
Type of reconstruction					
osteoarticular allograft	8	32	38	30	0.20
prosthesis	11	44	73	58	
allograft prosthesis composite	6	24	14	11	
Operated on shoulder before reconstruction					
yes	4	16	18	14	0.76
no	21	84	107	86	
Institution					
hospital 1	20	80	91	73	0.62
hospital 2	5	20	34	27	
Primary vs. metastatic disease					
primary	21	84	40	32	<0.001
metastatic	4	16	85	68	
Revision surgery on shoulder performed					
yes	8	32	24	19	0.18
no	17	68	101	81	

	median (IQR)	**range**	**median (IQR)**	**range**	**P-value**
Size resection, cm	15 (11-16)	9.0-30	11 (9.0-15)	0.0-28	0.0099
Number of reoperations	0.0 (0.0-1.0)	0.0-4.0	0.0 (0.0-0.0)	0.0-6.0	0.18
Age at time of reconstruction	52 (40-64)	14-82	57 (44-66)	9.5-82	0.41
Follow-up time, years	7.1 (4.5-14)	1.0-40	1.5 (0.33-5.5)	0.0082-28	<0.001

	mean ± SD,	**range**	**mean ± SD**	**range**	**P-value**
Size resection, cm	15 ± 5.0	9.0-30	12 ± 4.9	0.0-28	0.0052
Number of reoperations	0.8 ± 1.3	0.0-4.0	0.6 ± 1.4	0.0-6.0	0.51
Age at time of reconstruction	51 ± 19	14-82	53 ± 19	9.5-82	0.48
Follow-up time, years	11 ± 9.1	1.0-40	3.9 ± 5.2	0.0082-28	<0.001

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DUTCH SUMMARY (NEDERLANDSE SAMENVATTING)

Deel I - Inleiding en beschrijving chondrosarcomen

Hoofdstuk 1.

Algemene introductie, doelstellingen en opbouw van dit proefschift.

Chondrosarcomen zijn relatief veelvoorkomende primaire maligne bottumoren, waarvan het klinische gedrag nauw samenhangt met de histologische graad. Met name hooggradige chondrosarcomen (graad 2 en 3) en gededifferentieerde chondrosarcomen zijn agressieve tumoren met een slechte prognose en een hoog risico op metastasering. Vanwege deze slechte prognose is er in het bijzonder voor hooggradige conventionele en gedifferentieerde chondrosarcomen behoefte aan effectievere (systemische) behandelingen. Tot op heden is een chirurgische resectie de primaire behandeling, aangezien bestraling en chemotherapie beperkt effect hebben op zowel conventionele als gededifferentieerde chondrosarcomen. Daarom hebben wij ons in dit proefschrift gericht op immunotherapeutische aanknopingspunten die richting kunnen geven aan toekomstige effectieve systemische behandelingen.

Daarnaast hebben we de funtionele uitkomsten geëvalueerd na oncologische resecties van bottumoren rond de schouder en daaropvolgende reconstructies. We hebben ons op de schouder gericht aangezien dit een van de meest voorkomende locaties is voor zowel conventionele als gedifferentieerde chondrosarcomen. We hebben de volgende reconstructies van de proximale humerus vergeleken: endoprothesen, osteoarticulaire allografts en allograft prothese composite. We hebben deze reconstructies vegeleken met betrekking tot het beste functionele resultaat, implantaatoverleving en complicaties. Ten slotte hebben we zowel de functionele en oncologische uitkomsten onderzocht van patiënten die een chirurgische resectie hebben ondergaan voor chondrosarcomen in het schouderblad en sleutelbeen.

Hoofdstuk 2.

De Identificatie van Prognostische Factoren en de Overlevingsstatistieken van Conventionele Centrale Chondrosarcomen.

Het doel van deze studie was om de overlevingskenmerken van conventionele en gededifferentieerde chondrosarcomen weer te geven en daarbij onafhankelijke voorspellende factoren voor duur van overleving na ziekte te identificeren voor beide typen chondrosarcomen. Deze systematische review omvatte 13 studies met een totaal van 1114 patiënten. We hebben hierin aangetoond dat de prognose voor histologisch laaggradige tumoren over het algemeen goed is. Daarentegen zagen we een slechte overleving voor patiënten met hooggradige conventionele chondrosarcomen en gededifferentieerde chondrosarcomen. De specifieke

geïdentificeerde prognostische factoren voor een slechte overleving bij conventionele chondrosarcomen zijn; hooggradige tumoren, tumoren in het axiale skelet en tumoren in het bekken. Bij gedifferentieerde chondrosarcomen hebben we het percentage gedifferentieerde component in de tumor geïdentificeerd als een voorspellende factor voor ziektevrije overleving. Deze studie benadrukt dat er behoefte is aan onderzoeken naar nieuwe behandelingmogelijkheden, die de overleving van patiënten met chondrosarcomen positief beïnvloeden.

Deel II - Immunotherapeutische aanknopingspunten voor de behandeling van chondrosarcomen

Hoofdstuk 3.

Hoge infiltratie van tumor-infiltrerende lymfocyten (TIL), HLA en Immune Checkpoint Expressie bij Conventionele Hooggradige en Gedifferentieerde Chondrosarcomen en een Slecht Klinisch Beloop van de Ziekte.

Het doel van dit onderzoek was om de infiltratie van immuuncellen in chondrosarcomen aan te tonen en daarnaast de expressie van relevante immuunmoleculen te onderzoeken. We hebben een zelfontwikkelde tissue microarray geanalyseerd met 52 conventionele en 24 gedifferentieerde chondrosarcomen. Door middel van immunohistochemie hebben we de expressie van verschillende parameters geanalyseerd, welke zijn geassocieerd met tumorantigeen-specifieke immuunresponsen. In dit onderzoek lag de focus op CD4+ en CD8+ tumor-infiltrerende lymfocyten (TIL) en de expressie van HLA klasse I (zware keten), bèta-2-microglobuline, HLA klasse II en immune checkpoint moleculen B7-H3 en PD-1/PD-L1. Vervolgens hebben we de resultaten van deze kleuringen gecorreleerd aan de histopathologische kenmerken en de klinische uitkomsten van patiënten.

We hebben CD8+ TIL geïdentificeerd bij respectievelijk 21% van de conventionele chondrosarcomen en 90% van de gedifferentieerde chondrosarcomen. B7-H3 kwam tot expressie in 69% van de conventionele chondrosarcomen en in 96% van de gedifferentieerde chondrosarcomen. De expressie van PD-1 en PD-L1 werd respectievelijk geïdentificeerd in 53% en 33% van de geteste gedifferentieerde tumoren. Daarnaast hebben een verband aangetoond tussen de expressie van PD-L1 en een kortere tijd tot metastasering bij gedifferentieerde chondrosarcomen.

Dit onderzoek laat zien dat chondrosarcomen immunogene tumoren zijn, zoals blijkt uit de aanwezigheid van tumor-infiltrerende lymfocyten. Echter, de defecten in HLA klasse I antigeen expressie en de expressie van de immune checkpoint moleculen B7-H3 en PD-1/PD-L1 laten zien dat chondrosarcomen mechanismen gebruiken om immuunherkenning en daaropvolgende vernietiging te voorkomen. Deze resultaten

kunnen bijdragen aan het ontwerpen van immunotherapeutische strategieën voor de behandeling van chondrosarcomen. Met name therapieën gericht op B7-H3 en PD-1/PD-L1 kunnen effect hebben op deze maligniteiten, omdat ze de immunosuppressieve omgeving veroorzaakt door B7-H3 en PD-1/PD-L1 kunnen tegengaan.

Dit onderzoek toont tevens aan dat er behoefte is aan immunotherapeutische benaderingen die niet afhankelijk zijn van de expressie van HLA klasse I, aangezien de expressie van HLA klasse I antigeen verminderd is bij 77-92% van de conventionele chondrosarcomen.

Hoofdstuk 4.

Chondroitin Sulfate Proteoglycan 4-expressie in Chondrosarcomen: een potentieel doelwit voor immunotherapie.

In dit onderzoek richtten we ons op Chondroitin Sulfate Proteoglycan 4 (CSPG4) als doelwit voor een specifike op antilichamen-gebaseerde behandeling voor chondrosarcomen. CSPG4 is een proteoglycaan op het celoppervlak dat sterk tot expressie komt in verschillende maligniteiten en een beperkte distributie heeft in gezond weefsel. Hierdoor is CSPG4 een aantrekkelijk doelwit voor op antilichamen-gebaseerde therapie. We hebben de CSPG4-specifieke chimere antigeenreceptor (CAR) T-celtherapie onderzocht, een mechanisme voor het doden van tumorcellen welke onafhankelijk is van HLA klasse I antigeen expressie of functionaliteit.

Met behulp van onze tissue microarray hebben we primaire conventionele en gededitferentieerde chondrosarcomen immunohistochemisch gekleurd met CSPG4-specifieke monoklonale antilichamen (76 patiënten). Daarnaast hebben we 2 chondrosarcoom cellijnen geïncubeerd met CSPG4-specifieke CAR T-cellen om de effectiviteit van deze therapie bij het doden van chondrosarcoom cellen *in vitro* te evalueren.

In conventionele chondrosarcomen zagen we een matige tot hoge expressie van CSPG4 bij respectievelijk 29 van de 41 (71%) tumoren. Bij gededitferentieerde chondrosarcomen identificeerde we bij 3 van de 20 (15%) tumoren een matige tot hoge CSPG4 expressie. Daarbij toonden we een positieve associatie aan tussen CSPG4-expressie en de tijd tot metastasering bij beide subtypes. In de 2 met CSPG4 CAR T behandelde cellijnen vond er celdood plaats van respectievelijk >80% en 70% van de chondrosarcoomcellen.

We tonen aan dat op CSPG4-gerichte CAR T-cellen effectief zijn in het doden van CSPG4-positieve chondrosarcomen en mogelijk een effectieve aanvullende therapie kunnen zijn bij CSPG4-positieve conventionele en gedifferentieerde chondrosarcomen. Deze strategie kan met name relevant zijn, omdat we eerder hebben aangetoond dat de

expressie van HLA klasse I frequent verminderd is bij conventionele chondrosarcomen en deze therapie onafhankelijk is van de expressie van HLA klasse I.

Deel III - Resultaten van oncologische resectie rond de schouder

Hoofdstuk 5.

Uitkomsten van de Reconstructie van de Proximale Humerus na Oncologische Resectie: Een Systematische Review.

In deze review was ons doel om te bepalen wat de beste manier is om de proximale humerus te reconstrueren na een oncologische resectie in verband met maligne of lokaal agressieve goedaardige bottumoren. De proximale humerus is een veelvoorkomende locatie van chondrosarcomen en andere tumoren van het appendiculaire skelet. Door het uitvoeren van een systematische review probeerden we te achterhalen welke chirurgische reconstructie het beste functionele resultaat geeft en de langste overleving van het implantaat en daarnaast het laagste complicatierisico heeft. De geïncludeerde chirurgische reconstructies waren endoprothesen, osteoarticulaire allografts en allograft prothese composiet. Onze systematische review omvatte 29 studies met een totaal van 693 patiënten. Vanwege de heterogeniteit en bias in de studies rapporteerden we onze resultaten op een beschrijvende manier.

In de onderzoeken met prothesen varieerden de functionele scores van 61% tot 77% (10 studies, 141 patiënten), gemeten met de Musculoskeletal Tumor Society (MSTS) score. In de osteoarticulaire allograft onderzoeken varieerden de functionele uitkomsten van 50% tot 78% (8 studies, 84 patiënten) en in de allograft prothese composiet onderzoeken varieerden de functionele scores van 57% tot 91% (10 studies, 141 patiënten).

De implantaat overleving varieerde in de studies voor prothesen tussen 0,38 en 1,0 (proportie, 341 patiënten). In de onderzoeken welke zich richtte op osteoarticulaire allograft varieerde de implantaat overleving van 0,33 tot 1,0 (proportie, 143 patiënten) en in de allograft prothese composiet groep varieerde de overleving van het implantaat tussen 0,33 en 1,0 (proportie, 132 patiënten).

Het totale absolute complicatiegetal per patiënt varieerde tussen 0,045 en 0,85 in de prothese groep. In de osteoarticulaire allograft onderzoeken varieerde het complicatiegetal tussen 0 en 1,5 en in de allograft prothese composiet onderzoeken varieerde het complicatiegetal per patiënt van 0,19 tot 0,79. Er was een hogere fractuurincidentie bij de osteoarticulaire allografts, maar andere complicaties waren vergelijkbaar.

Deze systematische review toonde overeenkomstige functionele resultaten en overlevingspercentages tussen de endoprothesen en de allograft prothese composiet, waarbij fracturen die worden waargenomen bij osteoarticulaire allografts leken te worden vermeden. Daarnaast geeft deze studie de noodzaak aan voor verdere samenwerking op het gebied van orthopedische oncologie door middel van vergelijkende onderzoeken om de superioriteit van een specifieke behandelingen te identificeren.

Hoofdstuk 6.

Functionele Resultaten en Complicaties na Oncologische Reconstructie van de Proximale Humerus.

In lijn met de eerder beschreven review hebben we onderzocht wat de beste methode is voor een gewrichtsreconstructie na chirurgische resectie van de proximale humerus. We selecteerden een cohort van 150 patiënten in 2 ziekenhuizen die een resectie van de proximale humerus ondergingen. Deze oncologische resectie werd gevolgd door de eerder genoemde oncologische reconstructies: endoprothesen, osteoarticulaire allografts en allograft prothese composiet.

We verzamelden prospectief gegevens van 25 patiënten in dit cohort, waarbij de QuickDASH onze belangrijkste uitkomstmaat was. Deze QuickDASH geeft een invaliditeitsscore van nul tot 100, waarbij een hogere score een grotere invaliditeit aangeeft. De gemiddelde Disabilities of the Arm, Shoulder and Hand (QuickDASH) vragenlijstscore was 26 in deze groep. Ter vergelijking, de gemiddelde DASH-score voor de Amerikaanse bevolking is 10 ± 15.

Er werden geen verschillen waargenomen tussen de 3 reconstructiemethoden wat betreft de QuickDASH-score, de Toronto Extremity Salvage Score voor de bovenste extremiteit, de Musculoskeletal Tumor Society-score voor de bovenste extremiteit en de Patient-Reported Outcomes Measurement Information System (PROMIS)-score.

Vervolgens hebben we retrospectief complicaties en de overleving van het implantaat beoordeeld. Fracturen, loslating van componenten en non-union van de reconstructie kwamen vaker voor in de osteoarticulaire allograft groep in vergelijking met de andere 2 groepen. We vonden geen verschillen in postoperatieve infecties, subluxaties en luxaties, migratie van de proximale humerus of zenuw- en wondcomplicaties tussen de verschillende reconstructietechnieken.

Deze studie toonde een algehele overleving van het implantaat van >50%, waarbij revisiechirurgie werd gebruikt als eindpunt voor onze overlevingsanalyse. Er was een hoger falingspercentage in de osteoarticulaire groep vergeleken met de andere 2 reconstructies.

De gerapporteerde functionele scores in ons functionele cohort (n = 21; gemiddelde, 64%; range, 53% tot 80%; IQR, 73% tot 87%) zijn vergelijkbaar met onze eerdere review (hoofdstuk 5). De complicatieratio's zijn ook vergelijkbaar wat betreft infecties, subluxaties, luxaties, proximale migratie van de humerus en vertraagde consolidatie. Deze grote serie bevestigt een hoger fractuurrisico bij osteoarticulaire allografts in vergelijking met de andere 2 methoden. Dit hogere fractuurpercentage verklaart het waargenomen hogere revisiepercentage en ogenschijnlijk lagere overlevingspercentage in deze groep vergeleken met de endoprothese- en allograft prothese composietgroep.

Hoofdstuk 7.

Functionele en Oncologisch Resultaten na Chirurgische Resectie van het Sleutelbeen en Schouderblad voor Primaire Chondrosarcomen.

In deze studie lag de focus op het functionele en oncologische resultaat van patiënten met een primair chondrosarcoom van het sleutelbeen en schouderblad die operatief werden behandeld. We selecteerden 20 patiënten met de diagnose van een primair chondrosarcoom van het sleutelbeen of schouderblad. Bij 18 patiënten bevond de tumor zich in het schouderblad en 2 patiënten hadden een chondrosarcoom van het sleutelbeen. We beoordeelden de functie van de patiënten aan de hand van de DASH en de PROMIS Physical Function - Upper Extremity. Het oncologische resultaat werd beoordeeld door de patiënten over tijd te volgen.

Bij de 18 patiënten met een chondrosarcoom van het schouderblad was de gemiddelde DASH-score 16 ± 16 en de gemiddelde PROMIS Physical Function - Upper Extremity score was 48 ± 10. We constateerden een betere functie bij patiënten met een intacte rotator cuff en glenoïd. Verder lieten we een verband zien tussen een verminderde overleving en metastases, lokale recidieven en een hogere tumor gradering.

PHD PORTFOLIO

Courses

- Brian Silber Spine Oncology Symposium – Massachusetts General Hospital (2014)
- MGH Clinical Research Program, the MGH Center for Human Genetic Research. (2013)
- Genetic Literacy: A Guide to Understanding the Language and Concepts of Modern Genetic Research (2013)
- A Primer on Complex Trait Genetics: Principles for the Clinical Investigator
- MGH Clinical Research Program and the MGH Biostatistics Center. (2013 & 2014)
- Problem-Based Biostatistics for the Clinical Investigator (2013)
- Applied Biostatistics for Clinical Trials (2013)
- Basic Biostatistics for Clinical Research (2013)
- Basic Biostatistics for Clinical Research: Working Sessions (2013)

Presentations

AMC orthopedic research meeting (2019)

S.P.F.T. Nota. Potential clinical relevance of immune checkpoints and HLA expression in chondrosarcoma.

Symposium AMC "Tutors & Phds" (2016)

S.P.F.T. Nota. Oncologisch onderzoek als Bostonian

Musculoskeletal Tumor Society meeting (2015, posters)

S.P.F.T. Nota, F. Sabbatino, V. Villani, V. Deshpande, G.P. Nielsen, J.H. Schwab, S. Ferrone.

Human Leukocyte Antigen (HLA) Expression And Immunological Events In Chondrosarcoma

S.P.F.T. Nota, T. Teunis, J. Kortlever, M. Ferrone, J. Ready, M. Gebhardt, K. Raskin, F. Hornicek, J. Schwab, S. Lozano-Calderon**.** Functional Outcome And Complications Following Oncologic Reconstruction Of The Proximal Humerus

Harvard Orthopaedic Trauma Research Day (2014)

D.P. ter Meulen, **S.P.F.T. Nota**, M.G.J.S. Hageman, D. Ring. Progression of heterotopic ossification around the elbow after trauma

Smith Day (2014)

D.P. ter Meulen, **S.P.F.T. Nota**, M.G.J.S. Hageman, D. Ring. Progression of heterotopic ossification around the elbow after trauma

MGH Clinical Research Day *(2014, posters)*

S.P.F.T. Nota, Y. Braun, D. Ring, J.H. Schwab. Incidence of Surgical Site Infection after Spine Surgery: The Impact of How Infection is Defined.

S.P.F.T. Nota, S.S. Patel, G.P. Nielsen, S. Ferrone, J.H. Schwab. Different HLA Class I antigen expression patterns in conventional and dedifferentiated chondrosarcoma.

Scientific Advisory Committee meeting (2014, poster)

T. Teunis, **S.P.F.T. Nota**, J.H. Schwab. Responsiveness of the corresponding author

Harvard Orthopaedic Trauma Research Day (2013)

S.P.F.T. Nota, S.A. Spit, T. Voskuyl, A.G.J. Bot, M.G.J.S. Hageman, D. Ring. The Correspondence

Between the Use Of Pain Medication And Patient Satisfaction After Orthopaedic Surgery.

Smith Day (2013)

S.P.F.T. Nota, A.G.J. Bot, D. Ring, P. Kloen. Disability Corresponds with Depression after Orthopaedic Trauma in The Netherlands.

the New England Hand Society (NEHS) Annual Meeting (2013)

A.C. Doring, **S.P.F.T. Nota**, Michiel G.J.S. Hageman, David Ring. The correlation between PROMIS Physical Function – Upper Extremity and QuickDASH in patients with upper extremity illness.

MGH Clinical Research Day *(2013, posters)*

T. Teunis, **S.P.F.T. Nota**, J.H. Schwab, F.J. Hornicek, S.A. Lozano-Calderón. Surgical reconstruction of the proximal humerus after tumor resection: a systematic review and meta-analysis.

S.S. Patel. **S.P.F.T. Nota**, S. Ferrone, J.H. Schwab. Lymphocytic infiltration and immune escape mechanisms in human chordoma

Teaching

Journal Club Hand Service Harvard (MGH)

Tutoring / Mentoring / Supervising research students (7x)

Clinical education for medical students.

Grants

HLA antigen defects and lymphocyte infiltration in chordoma. Chordoma Foundation.

- Granted: $50.000

PUBLICATIONS

This Thesis

S.P.F.T. Nota, D. O. Osei-Hwedieh, D. L. Drum, X. Wang, F. Sabbatino, S. Ferrone, J.H. Schwab. Chondroitin Sulfate Proteoglycan 4 expression in Chondrosarcoma: a potential target for antibody-based immunotherapy. *Front Oncol.* 2022 Aug

S.P.F.T. Nota, A. Al-Sukaini, S.S. Patel, F. Sabbatino, G.P. Nielsen, V. Deshpande, J.H. Yearley, S. Ferrone, X. Wang, J.H. Schwab. High TIL, HLA, and Immune Checkpoint Expression in Conventional High-Grade and Dedifferentiated Chondrosarcoma and Poor Clinical Course of the Disease. *Front Oncol.* 2021 Apr

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S.P.F.T. Nota, M.J.A.M. Russchen, F.J. Hornicek F, H.J. Mankin, K.A. Raskin, J.H. Schwab. Functional and oncological outcome of surgical resection of the clavicle and scapula for primary chondrosarcoma. *Musculoskelet Surg.* 2016 Nov

S.P.F.T. Nota, Y. Braun, J.H. Schwab, C.N. van Dijk, J.A. Bramer. The Identification of Prognostic Factors and Survival Statistics of Conventional Central Chondrosarcoma. *Sarcoma*. 2015 Nov

T. Teunis, **S.P.F.T. Nota**, J.H. Schwab, F.J. Hornicek, S.A. Lozano-Calderón. Outcome After Reconstruction of the Proximal Humerus for Tumor Resection: A Systematic Review. *Clin Orthop Relat Res.* 2014 Jul

Other publications

S.N. van Laarhoven, **S.P.F.T. Nota**, G.G. van Hellemondt, B.W. Schreurs, A.B. Wymenga, P.J.C. Heesterbeek. Association between postoperative zonal fixation of hybrid fixated tibial components in revision total knee arthroplasty and subsequent aseptic loosening: Appropriate metaphyseal fixation is key. *Submitted.* 2024 Feb

S.S. Patel, **S.P.F.T. Nota**, F. Sabbatino, G.P. Nielsen, V. Deshpande, X. Wang, S. Ferrone, J.H. Schwab. Defective HLA Class I Expression and Patterns of Lymphocyte Infiltration in Chordoma Tumors. *Clin Orthop Relat Res.* 2021 Jun

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S.T. Meijer, N.R. Paulino Pereira, **S.P.F.T. Nota,** M.L. Ferrone, J.H. Schwab, S.A. Lozano Calderón. Factors associated with infection after reconstructive shoulder surgery for proximal humerus tumors. *J Shoulder Elbow Surg.* 2017 Jan

Y. Braun, O. Van Wulfften Palthe, S. Janssen, **S.P.F.T. Nota,** B. Verbeek, J.H. Schwab. Interobserver Agreement in Measuring the Neural Foramina Using a 360-Degree Ultrasound: A Human Cadaver Study. *The spine journal.* 2016 Oct

C.M. O'Connor, Y. Braun, **S.P.F.T. Nota,** T. Baloda, D. Ring. The Association of Complementary Health Approaches With Mood and Coping Strategies Among Orthopedic Patients. *Hand (N Y).* 2016 Sep

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T. Teunis, **S.P.F.T. Nota**, J.H. Schwab. Do Corresponding Authors Take Responsibility for Their Work? A Covert Survey. *Clin Orthop Relat Res*. 2015 Feb

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S.P.F.T. Nota, J.A. Strooker, D. Ring. Differences in response rates between mail, e-mail and telephone follow-up in hand surgery research. *Hand (N Y).* 2014 Dec

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CONTRIBUTIONS CO-AUTHORS

Chapter 1. General introduction, aims and outline of this thesis

Concept and design: All authors.

Drafting of the manuscript: Sjoerd Nota.

Critical revision of the manuscript: All authors.

Final approval of the version to be published: All authors.

Chapter 2. The Identification of Prognostic Factors and Survival Statistics of Conventional Central Chondrosarcoma.

Concept and design: All authors.

Acquisition, analysis and interpretation of the data: All authors.

Drafting of the manuscript: All authors.

Critical revision of the manuscript: All authors.

Final approval of the version to be published: All authors.

Chapter 3. High TIL, HLA, and Immune Checkpoint Expression in Conventional High-Grade and Dedifferentiated Chondrosarcoma and Poor Clinical Course of the Disease.

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Acquisition, analysis and interpretation of the data: All authors.

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Final approval of the version to be published: All authors.

Chapter 4. Chondroitin Sulfate Proteoglycan 4 expression in Chondrosarcoma: a potential target for antibody-based immunotherapy.

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Drafting of the manuscript: Sjoerd Nota.

Critical revision of the manuscript: All authors.

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Chapter 5. Outcome After Reconstruction of the Proximal Humerus for Tumor Resection: A Systematic Review.

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Critical revision of the manuscript: All authors.

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Chapter 6. Functional Outcomes and Complications After Oncologic Reconstruction of the Proximal Humerus.

Concept and design: All authors.

Acquisition, analysis and interpretation of the data: Sjoerd Nota, Teun Teunis, Joost Kortlever and Santiago Lozano Calderon.

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Critical revision of the manuscript: All authors.

Final approval of the version to be published: All authors.

Chapter 7. Functional and oncological outcome of surgical resection of the clavicle and scapula for primary chondrosarcoma.

Concept and design: All authors.

Acquisition, analysis and interpretation of the data: All authors.

Drafting of the manuscript: Sjoerd Nota

Critical revision of the manuscript: All authors.

Final approval of the version to be published: All authors.

Chapter 9. General discussion and future perspectives

Concept and design: All authors.

Drafting of the manuscript: Sjoerd Nota.

Critical revision of the manuscript: All authors.

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ABOUT THE AUTHOR

Sjoerd Pieter Nota was born on the 25th of February 1987 in Apeldoorn and grew up in Nijmegen. After graduating from the Stedelijk Gymnasium in Nijmegen, he went to Amsterdam to study Medicine at the Vrije Universiteit Amsterdam in 2005.

Because of his interest in orthopedic surgery he focused on this field during his last clinical rotation at the former Kennemer Gasthuis. Subsequently, he started his research career with a research internship at the orthopedic department of the former Academic Medical Center (AMC). He obtained his Masters degree in 2012. Thanks to the well-established collaboration between AMC and the department of Orthopaedic Hand and Upper Extremity Surgery at Massachusetts General Hospital (MGH), he was given the opportunity to start doing research in Boston, USA for 2 years. While working at MGH he was involved in setting-up a new clinical research collaboration between the department of Orthopaedic Oncology in MGH and the orthopedic department of the AMC. Simultaneously, he was given the possibility to perform laboratory research in the Monoclonal Antibody & Immunotherapy Laboratory. After his return to Amsterdam, he continued to be involved in several research projects.

In 2015, Sjoerd Pieter started his residency in Orthopedic Surgery at the former AMC. As a resident he was trained in the OLVG, AmsterdamUMC, Tergooi MC and Amphia. After becoming an orthopedic surgeon in 2022, he continued specializing with a fellowship focusing on complex primary and revision arthroplasty of the knee and hip at the Sint Maartenskliniek in Nijmegen.

Currently, he is a fellow in revision surgery of the knee and hip at the Catharina Ziekenhuis in Eindhoven. Sjoerd Pieter lives in Amstelveen with Vera and their children Philip and Noor.