A quest through diagnostics and predictive tools and the exploration of new treatment strategies in clinical trials to improve cancer therapy

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The research described in this thesis was performed at the Division of Pharmacology and the Department of Clinical Pharmacology, Division of Medical Oncology of The Netherlands Cancer Institute, Amsterdam, the Netherlands.

Printing of this thesis was financially supported by:
Utrecht University
Oncology Graduate School Amsterdam

Lay out:
Kimberley M. Heinhuis

Cover:
Marga Heinhuis-van den Brink, Henk M. Heinhuis

Printed by:
Ridderprint

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De ontwikkeling van diagnostische en predicatieve hulpmiddelen en nieuwe behandelstrategieën in klinische studies om de behandeling van kanker te verbeteren.

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling ingevolge van het besluit van het college van promoties, in het openbaar te verdedigen op maandag 30 november 2020 des middags te 4.15 uur,

door

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geboren op 17 februari 1990 te Nieuwegein

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Preface.

Preface

The field of oncology is rapidly evolving with new knowledge, diagnostics and therapies. With the improvement of diagnostics tools, like more accurate scans and liquid biopsies, malignancies can be found at an earlier stage and a better sub-classification could be made[1]. This has led to a better understanding of tumor biology, more personalized care and improved the survival of cancer patients. The better understanding of tumor biology has also led to new targets for therapy, which resulted in the upcoming of targeted therapies and immunotherapy in cancer treatment.

Especially the introduction of checkpoint inhibitors has positively changed the landscape of cancer treatment with drastic effects in the survival of metastatic melanoma[1]. More and more new approaches are being explored, like new combinations of immunotherapy with chemotherapy or other immunotherapies, to enhance the efficacy and expand the indication of checkpoint inhibitors[2,3].

One downside of the introduction of these checkpoint inhibitors is the economic effect of these compounds on the healthcare costs. Therefore, new strategies are also investigated, like alternative dosing strategies and repurposing of drugs. "Older" off-patent agents are being repurposed for new indications to improve cancer treatment with less burden for health care costs. A well-known example is propranolol, which was originally registered for hypertension and is now being used to treat hemangioma[4].

This thesis will describe some examples of these different strategies to improve the treatment of cancer patients.

In **Chapter 1** we focus on the improvement of the diagnostics of sarcoma patients by investigating the applicability of circulating tumor RNA obtained from tumor-educated-thrombocytes as a liquid biopsy in sarcoma diagnostics.

Chapter 2 describes a tool to predict therapeutic response of sarcoma patients to different systemic therapies and radiotherapy. The ultimate goal of this study is to develop a strategy to predict which therapy a patient should receive, in order to prevent treatment with ineffective compounds and to avert unnecessary adverse events.

In **Chapter 3** we give an overview of the effects of chemotherapeutic compounds on the immune system and evaluate potential candidates as combination partners for checkpoint inhibitors in the treatment of patients with solid tumors.

Chapter 4 discusses two early clinical trials in patients with solid tumors in which checkpoint inhibitors are combined with new antibodies directed against costimulatory receptors of the T-cell. In **Chapter 4.1** we describe the clinical results of treatment with nivolumab combined with a costimulatory antibody directed against glucocorticoid-induced TNFR-related protein (GITR). In **Chapter 4.2** the combination of nivolumab, with or without ipilimumab, with or without the new costimulatory antibody directed against OX40 is described.

Chapter 5 discusses the dosing strategy of monoclonal antibodies used in oncology, which initially was based on the body-size-based strategy originally applied for cytotoxic agents. Fixed-dosing of monoclonal antibodies could be a more optimal strategy and is already registered for nivolumab and pembrolizumab. In **Chapter 5.1** we show the implementation of fixed-dosing of monoclonal antibodies used in oncology in Dutch hospitals. **Chapter 5.2** gives an analysis of the amount of saved vials and the correlated economic impact of the implementation of fixed-dosing of monoclonal antibodies in the Netherlands Cancer Institute.

Next to investigating new drugs we also initiated a trial in the neoadjuvant setting to repurpose the off-patent drug propranolol for the new indication, angiosarcoma. First, we performed a systematic review of the literature for the effect of neoadjuvant systemic treatment on the resection margins and survival of angiosarcoma patients (**Chapter 6.1**). Secondly, we initiated the proof of principle study in which we evaluate the efficacy of propranolol in the treatment of angiosarcoma in the neoadjuvant clinical setting (**Chapter 6.2**).

Furthermore, we also searched for new targets in the treatment of sarcoma. **Chapter 7** describes potential new targets derived, from proteomics and sequence data, for sarcoma treatment, like PARP-1 and ALDH1A1.

Finally, a summary of the conclusions of the combined results of this research will be described in **Chapter 8** and future perspectives and challenges will be discussed.

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Chapter 1
RNA-sequencing of tumor-educated platelets, a novel biomarker for blood-based sarcoma diagnostics.

CHAPTER 1

RNA-sequencing of tumor-educated platelets, a novel biomarker for blood-based sarcoma diagnostics.

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Published in Cancers 2020 June:12(6), 1372.
Article.

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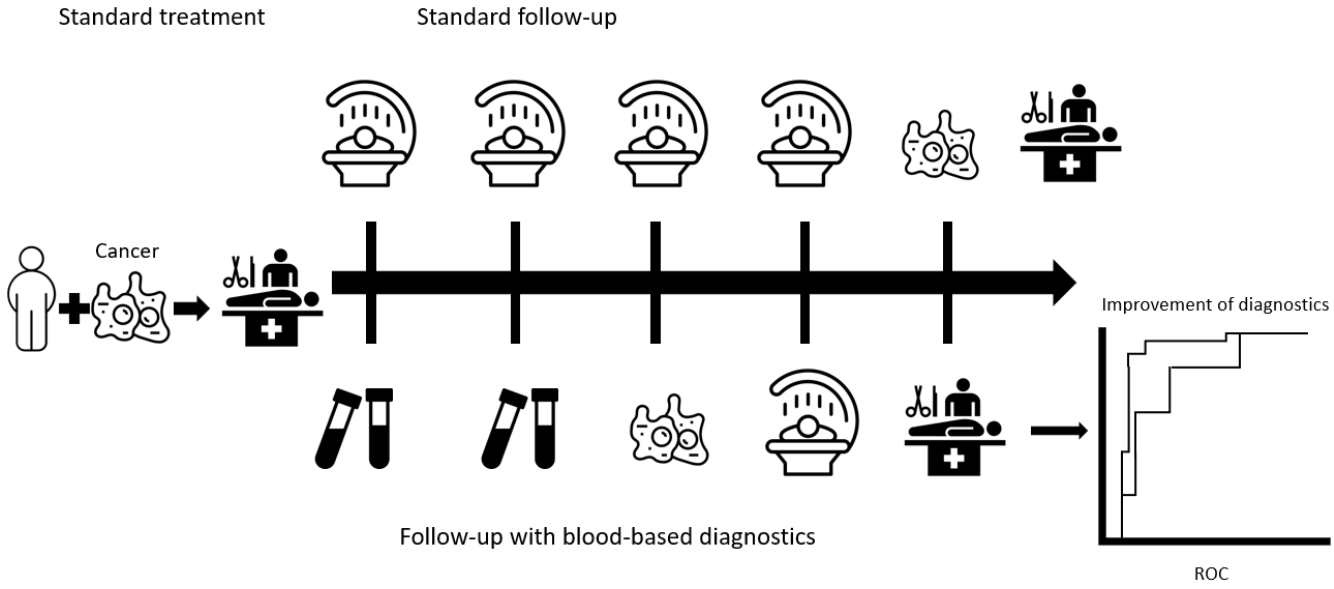
Abstract

Sarcoma is a heterogeneous group of rare malignancies arising from mesenchymal tissues. Recurrence rates are high and methods for early detection by blood-based biomarkers do not exist. Hence, development of blood-based liquid biopsies as disease recurrence monitoring biomarkers would be an important step forward. Recently, it has been shown that tumor-educated platelets (TEPs) harbor specific spliced ribonucleic acid (RNA)-profiles. These RNA-repertoires are potentially applicable for cancer diagnostics. We aim to evaluate the potential of TEPs for blood-based diagnostics of sarcoma patients. Fifty-seven sarcoma patients (active disease), 38 former sarcoma patients (cancer free for ≥3years) and 65 healthy donors were included. RNA was isolated from platelets and sequenced. Quantified read counts were processed with self-learning particle-swarm optimization-enhanced thromboSeq analysis and subjected to analysis of variance (ANOVA) statistics. Highly correlating spliced platelet messenger RNAs (mRNAs) of sarcoma patients were compared to controls (former sarcoma + healthy donors) to identify a quantitative sarcoma-specific signature measure, the TEP-score. ANOVA analysis identified distinctive platelet RNA expression patterns of 2,647 genes (false discovery rate <0.05) in sarcoma patients as compared to controls. The self-learning algorithm reached a diagnostic accuracy of 87% (validation set only; n=53 samples, area under the curve (AUC): 0.93, 95%-confidence interval (CI): 0.86-1). Our data indicates that TEP RNA-based liquid biopsies may enable for sarcoma diagnostics.



Introduction

Sarcoma is a malignancy arising from the connective tissue or bones, with an incidence of less than 1% in adults and 20% in children [1,2]. Sarcoma is a heterogeneous group of cancers with over 70 different histologic subtypes, and can arise throughout the whole body [1,3]. Due to a lack of tumor specific symptoms there is often a delay in diagnosis [2]. Approximately 25% of all sarcoma patients will develop distant metastases, rising up to 40-50% for sarcoma with high risk features [4,5]. Therefore, currently, most sarcoma patients are being followed for up to 10 years in order to detect recurrences. Early detection of low stage disease in the primary setting is associated with a better outcome, caused by improved resectability of the tumors, but lead-time bias should be taken into account [1,6]. Accurate diagnostic tools to achieve a correct and early diagnosis are important to potentially improve survival of sarcoma patients. Whereas in many cancer types blood-based markers have been developed to screen for disease recurrence, such as carcinoembryonic antigen (CEA) for colorectal cancer and prostate specific antigen (PSA) for prostate cancer [7,8], there are no clinically implemented tumor markers for sarcoma. Blood-based biomarkers have several advantages, including the low cost of screening, the relatively low patient burden compared to imaging and the lack of radiation exposure in the screening program. Also, follow-up with a low-invasive diagnostic tool instead of the more invasive screening program, which is currently applied, could improve the quality of life of sarcoma patients and an early detection method increases the chance of a better survival [6].

The applicability of several biomarkers in blood-based diagnostics are currently investigated, but most approaches show a lack in specificity in diagnosing the primary tumor [9,10]. A technique which can potentially overcome this problem of specificity is the blood-based diagnostic tool which analyses tumor-educated platelets (TEPs) [9,11–13]. Blood platelets are widely known for their role in hemostasis. However, recent research has revealed their contribution to the progression and metastasis of cancer [14], including in sarcomas [15–18]. Blood platelets contain messenger ribonucleic acids (mRNAs) which may undergo specific splice events in response to external stimuli, potentially induced by the primary tumor [9]. Such external queues may result in tumor-specific RNA-profiles that may be employed for blood-based cancer diagnostics and monitoring of tumor recurrence [9,11]. Previously, a self-learning thromboSeq algorithm has been employed to detect several forms of cancer, such as non-small-cell lung cancer, colorectal cancer, glioblastoma, breast cancer, pancreatic cancer and hepatobiliary cancer [9,11,19,20]. The aim of this study was to investigate whether TEPs could be used as a blood-based diagnostic tool in the detection of sarcoma patients.

Results

We included 57 patients with active sarcoma disease, and 103 controls of which 38 were former sarcoma patients (at least three years free of cancer and anti-cancer treatment) and 65 were individuals reported to have no cancer (healthy donors).

To prevent potential confounding effects of the variables age and gender, both series were matched [21]. The demographic characteristics are summarized in *Table 1* and the distribution of the histologic subtypes of the sarcoma series are provided in *Table 2*. The majority of sarcoma patients had metastatic disease (68%) and the most prevalent histological subtypes included were liposarcoma, gastrointestinal stromal tumor and leiomyosarcoma. We were unable to calculate a correlation between disease-stage and TEP-score because of the limited number of stage II samples (n=2).

In total, following filtering and quality steps (*Supplementary Figure S1*, *available at Cancers online*), 3,799 RNAs with sufficient read coverage were identified in the platelet profiles. To circumvent potential cell-free DNA contamination, the thromboSeq pipeline only includes spliced RNA reads (reads from exon-to-exon or intron-spanning, also termed 'spliced junctions') for downstream analyses. We first compared all sarcoma patients (n=57) to all controls (n=103) by analysis of variance (ANOVA) analysis, resulting in 2,647 RNAs with differential expressed spliced junctions (false discovery rate (FDR)<0.05; *Table S1*). Unsupervised clustering of particle-swarm optimization (PSO)-enhanced FDR selection (2,537 RNAs, FDR <0.033) resulted in clear separation of sarcoma patients and controls (*Figure 1a*, $p<1.6x10^{-6}$). Hence, we concluded that TEP RNA is significantly altered in patients with sarcoma as compared to healthy donors and patients with no active disease, and resulted in a differentially expressed spliced junction RNA signature, and resulted in a differentially expressed spliced junction RNA signature.

Subsequently, we developed a self-learning classification algorithm that enables for independent diagnosis of patients with sarcoma. For this, we separated the complete dataset into training, evaluation, and validation series. Here, the training series is employed for biomarker panel selection and training of a self-learning support vector machine (SVM)-algorithm. Subsequently, the performance of this compiled biomarker panel and SVM-algorithm was evaluated in the evaluation series after which new instructions regarding biomarker panel selection thresholds were provided to the training series using particle-swarm optimization. This process was performed 1,000 times in order to improve classification accuracy in the evaluation series. Once satisfied, the algorithm was locked and an independent set of validation samples was classified. The training series consisted of 21 sarcoma samples and 34 controls, the evaluation series consisted of 19 sarcoma samples and 33 controls, the validation series consists of 17 sarcoma samples and 36 controls (*Table 1*). The optimization process resulted in an optimum detection accuracy of 90% in the evaluation series, applying the default cutoff of the TEP-score of 0.5 (number (n)=52 samples, area under the curve (AUC): 0.94, 95%-confidence interval (CI) 0.87-1, *Figure 1b*, red line), and a detection accuracy of 88% in the validation series (n=53 samples, AUC: 0.93, 95%-CI: 0.86-1, p<0.001, *Figure 1b*, blue line). Post-hoc evaluation of the training series using a leave-one-out cross validation (LOOCV) approach resulted in similar detection rates (accuracy: 85%, AUC: 0.92, 95%-CI 0.88-1, *Figure 1b*, grey line). In order to assess uniqueness of sarcoma signature, we performed venn diagram analysis of this signature as compared to the signature identified in Best et al. Cancer Cell 2017 [11] (non-small-cell lung

cancer (NSCLC) versus non-cancer controls) and Best et al. Nature Protocols 2019 [20] (lower-grade glioma (LGG) versus controls).

Table 1. Demographic characteristics. The demographic characteristics of the sarcoma and controls (former sarcoma patients and healthy donors) are shown, and their distribution between the training, evaluation and validation series. F=female, IQR=interquartile range, M= male, N=number of patients

	Training		Evaluation		Validation	
Sarcoma cohort N	21		19		17	
Median age (IQR) in years	56 (19)		60 (18)		60 (19)	
F/M %	33/67		74/26		35/65	
Localized N (%)	5 (24%)		9 (47%)		4 (24%)	
Metastasized N (%)	16 (76%)		10 (53%)		13 (76%)	
Controls cohort N	34		33		36	
Median age (IQR) in years	61 (20.5)		59 (18)		54 (26.5)	
F/M %	55/45		85/15		58/42	
Former sarcoma /healthy donors N	11	23	10	23	12	24
Median age (IQR) in years	72 (13.5)	57 (23)	59.5 (17)	59 (17)	64 (11.5)	48 (22.5)
F/M %	55/45	100/0	50/50	100/0	58/42	100/0

Table 2. Overview of histologic subtypes of the included sarcoma patients. Distribution of the different histologic subtypes of sarcoma patients between the training, evaluation and validation series.

	Training	Evaluation	Validation
Dedifferentiated liposarcoma	3	1	2
Myxoid liposarcoma	3	2	1
Pleomorphic liposarcoma	1	1	0
Leiomyosarcoma	3	3	5
Gastrointestinal Stromal Tumor	5	5	6
Myxofibrosarcoma	1	2	0
Undifferentiated pleomorphic sarcoma	1	1	1
Others			
Angiosarcoma	1	1	0
Ewing sarcoma	1	0	1
Extraskeletal chondrosarcoma	0	0	1
Malignant peripheral nerve sheath tumor	1	1	0
Synovial sarcoma	1	2	0
Total	21	19	17

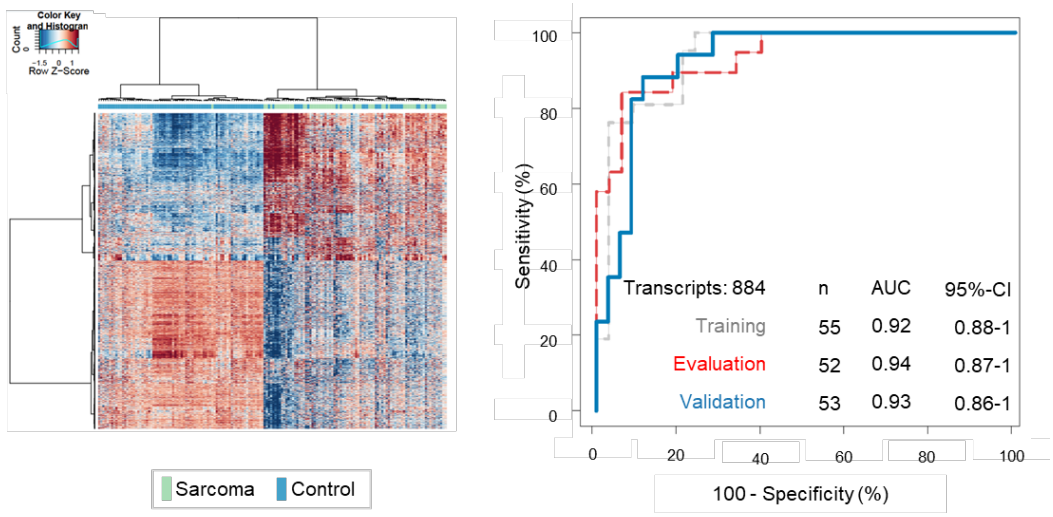
We observed in total an overlay of 66 spliced RNAs between all three signatures, whereas the majority appears to be uniquely present in any of the signatures (for

LGG: 1168 / 1711 (68%); for NSCLC: 533 / 1000 (53%); for sarcoma: 472 / 824 (57%), *Supplemental Figure S2, available online at Cancers*). Hence, we conclude that an unique sarcoma signature can be selected from TEP RNA profiles. A variant of our sarcoma classifier trained on the subset of 472 sarcoma specific transcripts as visualized in *Supplemental Figure S2* (*available online at Cancers*) did not outperform the original swarm-enhanced biomarker panel of 884 transcripts.

To provide better insight in the classification of patients with sarcoma versus controls, we determined the distribution of the TEP-scores of the different samples in the training, evaluation and validation series (*Figure 2*). The TEP-score ranges from zero to one and represents the algorithms' measure of the expression of the sarcoma profile in a particular sample. A TEP-score of 0.5 was used as a cut-off value for an adequate differentiation between the diagnosis sarcoma and the diagnosis healthy. The cut-off value was based on the most accurate fit with a high sensitivity, specificity and accuracy. Because the aim of our study is to evaluate the potential of TEPs in sarcoma diagnostics, the exact cut-off of 0.5 was selected to obtain high specificity to avoid an excess of false-positive samples. The default cutoff of the SVM algorithm (0.5) resulted in a specificity of 88%, sensitivity of 81% and accuracy of 85% for the training series, a specificity of 94%, sensitivity of 84% and accuracy of 80% for the evaluation series, and a specificity of 86%, sensitivity of 88% and accuracy of 87% for the validation series.

The histologic subtypes of the nine sarcoma patients who were wrongly classified as control (in either the training, evaluation or validation series) were: four patients with locally-advanced or metastasized dedifferentiated liposarcoma; two patients with metastasized leiomyosarcoma; two patients with small lesions of gastrointestinal stromal tumor; and one patient with localized myxoid liposarcoma. Four of these patients had stable disease. The tumor size and tumor stage of the outliers was not different from the
correctly classified sarcoma patients. The histologic subtypes of the eleven controls who were wrongly classified as sarcoma (in either the training, evaluation and validation series) were: one healthy donor; three patients with fibrosarcoma; three patients with myxoid liposarcoma; two patients with gastrointestinal stromal tumor; one patient with angiosarcoma; one patient with an alveolar soft part sarcoma in their medical history. None of the wrongly classified former sarcoma patients had a recurrence at their next follow-up visit with a median follow-up time of 24.7 months after the blood draw.

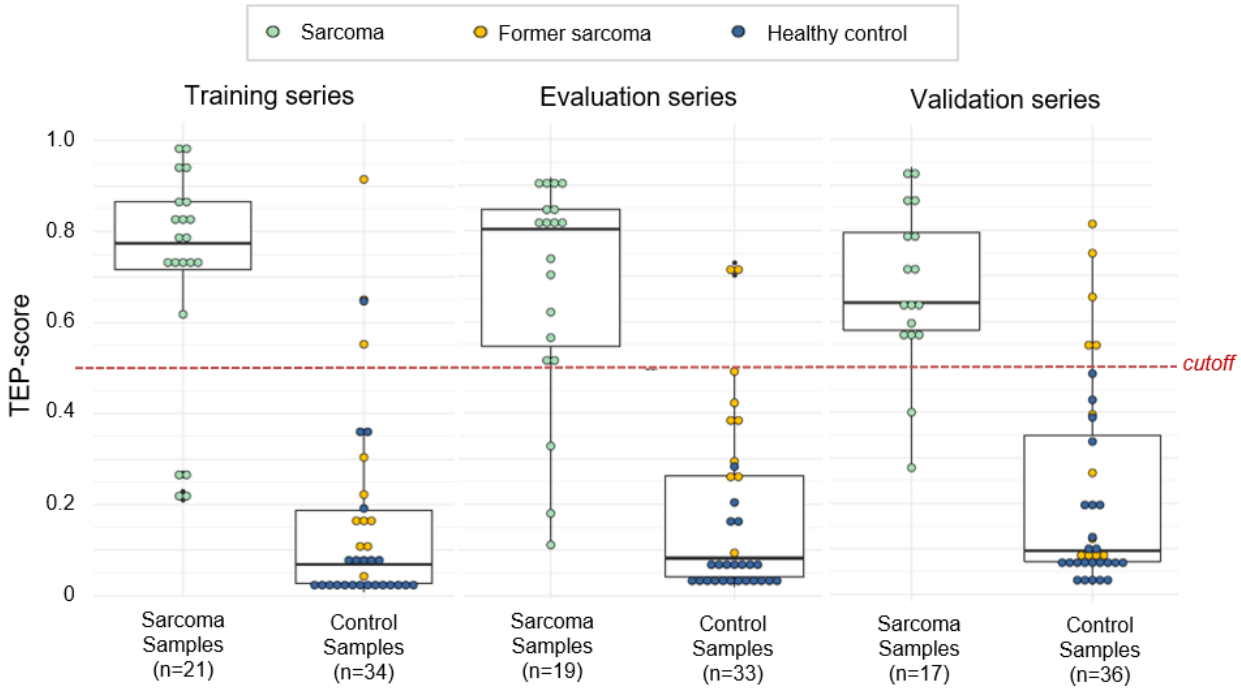
Figure 1. TEP thromboSeq analysis of sarcoma patients versus controls. Figure 1A. Particle-swarm optimization (PSO) optimized heatmap of sarcoma patients versus controls. Out of 3,799 spliced transcripts detected in the blood platelets, 2,647 transcripts were differentially expressed spliced junctions(false discovery rate <0.05) and 2,537 transcripts were used for clustering analysis (false discovery rate <0.033). Unsupervised hierarchical clustering of differentially expressed spliced junctions ribonucleic acid (RNA) transcripts between controls (blue, number (n)=103) and sarcoma patients (green, n=57). The columns indicate the different patient samples. The rows indicate the differentially expressed spliced junction RNA transcripts. The color intensity represents the Z score-transformed RNA expression value. **Figure 1B** Receiver operating characteristics (ROC) analysis of the PSO-enhanced thromboSeq classifications using controls, including healthy donors and former sarcoma patients, and patients with sarcoma. The red dashed line indicates the ROC of the evaluation series (n=52), classified by the self-learning SVM algorithm developed with 884 transcripts and spliced RNA levels derived from the training series. The grey dashed line indicates the post-hoc assessment of the 884 transcripts by leave-one-out-cross-validation (LOOCV) on the training series (n=55). The blue line indicates the ROC of the validation series (n=53). As an internal control experiment, shuffled class labels (n=1,000) shows a median area under the curve (AUC) in the validation series of 0.47 and an interquartile range (IQR) of 0.36. Shuffled samples in training (1,000 iterations) resulted in a median AUC in the validation series of 0.92 with an IQR of 0.05.



(a) PSO optimized heatmap of sarcoma versus controls classification

(b) ROC analysis of the PSO-enhanced thromboSeq

Figure 2. Distribution of the TEP-score for the training, evaluation and validation series. The distribution of the tumor-educated platelets (TEP)-score for the training, evaluation and validation series for both discriminative groups, patients with sarcoma and controls. Samples are colored by their cancer status. In green, patients with present sarcoma tumor load, in blue, healthy donors without any history of cancer, in yellow, samples obtained from former sarcoma patients who are currently monitored for disease recurrence. Below are per series shown 2x2 cross-tables, indicated are sample numbers and detection rates in percentages.



Actual Class

predicted class	Sarcoma	Control	Total (n)
Sarcoma	17 (81%)	4 (12%)	21
Control	4 (19%)	30 (88%)	34
	21 (100%)	34 (100%)	55

Actual Class

predicted class	Sarcoma	Control	Total (n)
Sarcoma	16 (84%)	2 (6%)	18
Control	3 (16%)	31 (94%)	34
	19 (100%)	33 (100%)	52

Actual Class

predicted class	Sarcoma	Control	Total (n)
Sarcoma	15 (88%)	5 (14%)	20
Control	2 (12%)	31 (86%)	33
	17 (100%)	36 (100%)	53

Discussion

The goal of this proof-of-concept study was to investigate whether TEPs can be employed as a blood-based biomarker tool for sarcoma patients. We showed that TEP RNA is significantly altered in the presence of sarcoma as opposed to former sarcoma patients and healthy donors. With the developed TEP-score, most sarcoma patients could be identified and distinguished from control samples. Among the false negative sarcoma patient samples, there was no specific histologic sarcoma subtype more abundant.

We showed that thromboSeq might be a promising technique in sarcoma diagnostics, however its potential in monitoring of tumor recurrence still needs to be

explored. The follow-up of sarcoma patients, currently consists of physical examination and radiologic imaging every few months. Additional blood sampling for the TEP RNA-based analysis may be obtained during the standard follow-up visits to investigate if recurrences could be objectified in an earlier phase compared with radiologic imaging alone. Whether this screening method can be used for all sarcoma types remains to be investigated. Creating a single TEP score for sarcoma might be challenging due to the heterogeneity of the sarcoma population. However, there are currently no clinical relevant blood-based markers available for sarcoma patients, and therefore development of a new tool is warranted.

The various techniques for liquid biopsies which are currently under investigation for their application in sarcoma can be divided in four groups of biosources: circulating tumor cells (CTCs), circulating cell-free nucleic acids (ccfNAs), exosomes, and metabolites [15]. The advantage of CTCs is that multiple components can be investigated like DNA, RNA and proteins. Unfortunately, sarcoma patients only have a limited amount of CTCs in their bloodstream, and therefore a larger sample volume would need to be collected. Moreover, it seems hard to determine relevant aberrations in CTCs of sarcoma patients [10]. CcfNAs are considered to have a higher sensitivity than CTCs [22]. Exosomes can be measured in all kind of body fluids and are related to angiogenesis and metastasis, which makes them a promising biosource to predict tumor progression and metastasis. However, most exosomes lack tumor-specific markers [10]. Another biosource is composed of metabolites, which are considered representative of the tumor phenotype. Even though they may offer more detail on potential targets for therapy, these metabolites may also be quite sensitive to physiological and chemical changes of the environment [15]. Here, with the PSO-enhanced thromboSeq an AUC of 0.93 in a 53-samples validation series was achieved, which makes the TEP-score a potentially sensitive and specific tool.

TEPs have been tested in other cancer types as well, and the accuracy for sarcoma is comparable [9,11,19–21]. There are several limitations to this proof-of-concept study. Although we included sufficient sarcoma samples for an initial classification, and a separation between sarcoma and controls was observed, in the current analysis former sarcoma patients were pooled with the asymptomatic controls to have a better age- and gender-status-matched series. Despite the fact that former sarcoma patients classified more as control than as sarcoma (*Figure 2*), this creates a potential bias. Another limitation of this study is that we cannot yet differentiate between the different histologic subtypes and different stages of disease of sarcoma. To improve this ability of the algorithm, we need to include more patients per histologic subtype and with lower stage of disease in a prospective study. Finally,

analysis of the biological function of the altered spliced TEP transcripts is required to further understand the role of platelets in patients with sarcoma.

Materials and Methods

Inclusion of patients

Blood samples were collected from sarcoma patients with active tumor load (before or during anti-cancer treatment (sarcoma series). All samples were processed according the same standardized protocol of Best *et al.* [20]. Patients were ≥18 years, and written informed consent had to be obtained. All histologic subtypes of sarcoma were eligible. Two samples were collected at different time points in patient treatment in two cases in the sarcoma validation series. Samples of former sarcoma patients (defined as at least three years free of cancer and anti-cancer treatment) and healthy donors were also collected. Healthy donors reported to be without any type of cancer, currently or in the past. Former sarcoma patients and healthy donors were pooled in a control series, which was age- and gender-matched to the sarcoma patient sample series. The samples and associated clinical data of all individuals was collected and stored with a retraceable code, and fully anonymized.

This study was approved by the institutional review board of the Netherlands Cancer Institute under number CFMPB420.

Particle-swarm optimization-enhanced thromboSeq analysis

For the RNA extraction, sequencing and interpretation we used the standardized protocol of Best *et al.* [20]. In the first step the blood platelets were isolated from whole blood samples and the RNA was extracted and sequenced with SMARTer-based complementary deoxyribonucleic acid (cDNA) synthesis and amplification and Illumina TruSeq cDNA labeling and sequencing on the Illumina platform. All steps are quality-controlled by Bioanalyzer analysis. Secondly, the blood platelet RNA-sequencing data was processed and used for the development and validation of the PSO-enhanced classification algorithm. To circumvent potential cell-free DNA contamination, only intron-spanning spliced RNA reads were selected for analysis. ANOVA was used to determine the difference in the level of spliced RNAs between sarcoma patients and control samples. The panel resulting in the most optimal separation of the groups after unsupervised clustering was visualized in the PSO-enhanced heatmap according to the default settings as published before (12 iterations and 200 particles, 1200 particles in total). For PSO enhanced algorithm development, we applied the predefined settings; libsize correlation between -0.1 and 1.0, FDR between 0.00001 and 1.0, correlated transcripts between 0.5 and 1.0 and ranked transcripts between 200 and all 3,799 detected transcripts. We selected the particle (algorithm settings) showing the best performance on the evaluation series after creating 100 particles during 10 iterations (1,000 particles in total). The particle resulting into the highest AUC on the evaluation series was applied post-hoc on the training set (LOOCV) and on the validation set. A FDR-threshold of <0.05 was stated as statistically significant [9,11]. A sarcoma TEP-

score was generated as a measure for the probability of a sample belonging to the sarcoma cohort.

Conclusions

TEP-based liquid biopsies can potentially be used as a blood-based diagnostic tool for sarcoma patients. The PSO-enhanced thromboSeq analysis of TEPs is a highly sensitive and specific tool for the detection of sarcoma. Algorithm optimization by including more prospectively collected samples into the development process is likely to improve the reproducibility of the test. Hence, a prospective study is warranted.

Patents: M.G.B. and T.W. are inventors on relevant patent applications.

Supplementary Materials: The following are available online at https://doi.org/10.3390/cancers 12061372, Figure S1: Inter sample correlation, Figure S2 Venn diagram, Table S1: overview of differential expressed spliced junction RNAs and corresponding ANOVA results, Table S2 Swarm-enhanced biomarker-panel sarcoma.

Funding: This research received no external funding.

Acknowledgments: We would like to thank Anne Miek Koenen for her contribution in the identification and inclusion of the patients.

Conflicts of Interest: K.M.H., S.G.J.G.V., G.D., D.B., N.So., F.C., R.L.H., J.H., W.J.H., N.St. declare no conflict of interest. M.G.B. and T.W. are inventors on relevant patent applications. T.W. received funding from Illumina, Inc and is shareholder of GRAIL, Inc.

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Chapter 2

Development of a platform of patient-derived cell lines of soft tissue sarcoma: to predict treatment outcome.

CHAPTER 2

Development of a platform of patient-derived cell lines of soft tissue sarcoma: to predict treatment outcome.

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Interim analysis.

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Abstract

Background: Soft tissue sarcoma (STS) is extremely rare (incidence of <1% of all diagnosed malignancies) and heterogeneous (>100 histologic subtypes). Therefore, large randomized trials are not feasible and other approaches for personalized medicine are needed. A predictive model for individual treatment response, incorporating the biological tumor behavior, could improve prognosis and quality of life. The aim of the study is to evaluate response correlation of 2D primary tumor cell lines and clinical responses.

Methods: Patient derived sarcoma cell lines were grown and exposed to cytotoxic or targeted agents or radiotherapy. We compared two different culture methods, one based on fetal bovine serum (FBS) and one with autologous human derived serum (HS). Measured response of the cell lines was compared with the clinical response of the patient to investigate if the cell lines could be used to predict treatment outcome. Clinical response was defined according to RECIST 1.1 at the first response evaluation, while response in the cell lines was defined as percentage of viable cells after exposure to treatment. A growth inhibition of ≥50% after exposure to the standard IC50 value of the systemic compound or to 6 Gy in the cell lines was set as response to therapy.

Results: We achieved a success rate of 69% in establishing cell lines (n=22). Culturing in HS instead of FBS resulted in a growth advantage in eight out of sixteen cell lines, a comparable growth rate in three cell lines and inhibited growth in four cell lines, with a median difference of six days per cell lines. The correlation of response to therapy could be investigated in twelve cases for cytotoxic or targeted agents and in eleven cases for radiotherapy. Viability assays could be initiated after a median of seventeen days of culturing. We could predict a clinical response to systemic therapy in 67% (n=12) for FBS cultured cell lines and 71% for HS cultured cell lines (n=7). A clinical response to radiotherapy was associated with the response in the cell lines in 64% of the FBS cultured cell lines (n=11) and in 80% of the HS cultured cell lines (n=5).

Conclusions: HS can be used for culturing sarcoma cell lines instead of FBS. Clinical treatment response to systemic compounds or radiotherapy could more accurately be predicted in the HS cultured cell lines. Advantages of HS over FBS were the improved success rate of establishing stable cell lines, the enhanced growth rate and the slightly better correlation between the clinic and responses in the cell lines, which makes HS cultured cell lines a basis for further exploration of its role as (early) biomarker for response to drug an radiation treatment.

Introduction

Despite improved insights in the biologic behavior of malignancies and the introduction of new therapies in the treatment of soft tissue sarcoma, response rates

remain dismal. The published response rates vary enormously, from 10–50% depending on the used compounds, patient selection and histological subtype (1). A predictive model for individual treatment response, incorporating the biological behavior of the tumor, could improve prognosis and quality of life of patients with soft tissue sarcoma.

Soft tissue sarcomas arise from the mesenchymal tissue at any body site and are extremely rare, representing less than 1% of all newly diagnosed malignant tumors (2,3). The histopathologic spectrum of sarcomas is broad with over 100 histologic subtypes (2,4). A range of completely benign to aggressively malignant tumor categories are observed, all characterized by specific clinical behavior patterns ranging from indolent to highly aggressive with different sensitivities for systemic therapies. Therefore, large randomized trials are hardly feasible and other approaches for personalized medicine are needed (4).

In the current standard of care, surgery is the cornerstone in the treatment of non-metastatic sarcomas, often combined with (neo-)adjuvant radiotherapy and sometimes with (neo-)adjuvant systemic therapy (5). Patients with metastatic disease are usually treated with systemic agents, consisting of both the older cytotoxic chemotherapies, like doxorubicin and ifosfamide, and the newer targeted therapies, like the tyrosine kinase inhibitor pazopanib (6,7). However, this approach ignores the heterogeneous biological behavior of most sarcoma subtypes, which leads to worse prognoses and unnecessary toxicity for sarcoma patients (7).

Furthermore, given the vast diversity of sarcoma subtypes, it is also unlikely that a uniform treatment will be optimal for all sarcomas. An individual patient and/or histology tailored approach should translate in the best clinical benefit in terms of local control at the cost of minimal toxicity.

A possible strategy to predict response could be to use in vivo patient derived xenograft (PDX) models for drug sensitivity analysis. However, developing a PDX model is quite expensive and time consuming, since it usually takes months before reliable results are obtained (8,9). Another possibility is to use patient derived cell lines (8). In such an in vitro model, tumor material of sarcoma patients could be used to develop a 'classic' 2D cell line, in which the cells grow in a monolayer, or to develop a 3D cell line model, in which the cells form organoids. This 3D model could potentially reflect the patient situation even more accurate because these organoids reflect the natural microenvironment more closely, with a higher degree of structural complexity and homeostasis (8,10,11). However, sarcoma cell line models are scarce due to the rarity and heterogeneity of the disease and mainly consists of the most common histologic subtypes (8,12,13), and the development of sarcoma cell lines is challenging, with success rates ranging from 30 to 58% (9,14–16). Optimization of the culture

method could result in an enhanced success rate of establishing sarcoma cell lines. One possible strategy is to use autologous human derived serum (HS) instead of the standard fetal bovine serum (FBS). The rational for this adjusted culture method is that we hypothesized that all the necessary growth factors for tumor growth should be present in the blood of the patient itself (17).

To conclude, a predictive model for treatment response of a specific patient, which would also take the biological behavior into account, is warranted and could be used for a more personalized systemic treatment. The aim of the pilot study is to develop a platform of 2D and 3D primary cell cultures as a fast personalized drug and radiation sensitivity biomarker, in order to evaluate the optimal tool to enable early prediction of clinical responses of sarcoma patients to radiotherapy and systemic therapy.

Methods

Patient recruitment

The study population was accrued from the multidisciplinary sarcoma board from the participating center and consisted of patients with histological confirmed intermediate to high grade soft tissue sarcoma, both localized and metastasized disease, including local recurrences. Patients were eligible if they were ≥18 years of age, and if the localization of the tumor enabled a safe harvesting of tumor tissue via biopsy or surgery. The harvesting of tumor tissue was done at baseline prior to any anti-sarcoma therapy. All clinical data and corresponding cell lines were anonymized.

Culture method 2D cell lines

Tumor tissue was collected during surgery or biopsy and dissected into smaller pieces. The minced tumor pieces were incubated in 'digestion mix' at 37 degrees for 3-4 hours until the tumor tissue is completely dissolved into single cells. The digestion mix consisted of medium (DMEM glutmax nutrient mix from Gibco), 1% penicillin-streptomycin, 200 mg collagenase A and 1.25 mg DNase. Subsequently, the single cells were incubated in medium (DMEM glutmax nutrient mix from Gibco) in 6-well plates in a 37 degrees incubator.

Two culture methods were compared: the standard culture method with medium containing 10% (50 per 500mL) fetal bovine serum (FBS) and the adjusted culture method with medium containing 10% (40 per 400mL) autologous human derived serum (HS).

For the HS, eight serum-separating tubes of blood were obtained by the central laboratory, of the same patient who underwent surgery or a biopsy. The serum was

isolated and added to nutrition mix medium in a ratio of 10% (40 per 400mL). The cell lines were passaged when a coverage of 70-80% was achieved.

Culture method 3D cell lines

The method for the formation of the 3D cell lines was based on the article of Sachs et al. (18). After the digestion step, the singles cells were resuspended in Matrigel and plated as drops in non-adherent wells plates. After a solidification period of >30 minutes, medium was added to the well plates. We compared both the standard medium (FBS) and our adjusted medium (HS).

Viability assay systemic compounds

The human-derived cell lines were plated in 96-well plates. We compared both culturing methods (FBS and HS medium). After 24 hours of incubation the cell lines were exposed to the IC50 values of the different systemic therapies in triplo for 72h. The concentration of the different agents was based on IC50 values in literature (Table 1). The amount of viable cells was measured with a viability assay after the cells were incubated for four hours after staining with Cell Titer Blue (19). A growth inhibition of ≥50% at the IC50 concentration in the cell lines was set as cutoff for response (SD, PR or CR in clinic) (20).

Table 1. Overview of IC50 values in literature for the systemic agents used in this study (21)**.**

Systemic agent	IC50 concentration
Docetaxel	0.016 ug/mL
Doxorubicin	0.1 ug/mL
Ifosfamide	2.2 ug/mL
Melphalan	1 ug/mL
Paclitaxel	0.09 ug/mL
Pazopanib	0.04 ug/mL
Trabectidin	0.0006 ug/mL

Viability assay radiotherapy

The human-derived sarcoma cell lines were plated in 96-well plates and both culture methods (FBS and HS medium) were compared. After an incubation period of 24 hours the 96-well plates were exposed to 6 Gy. After nine days of incubation, a viability assay was used to quantify the amount of viable cells. A growth inhibition of ≥50% after exposure of the cell lines to 6 Gy, was set as cutoff for response, because this cutoff value showed the best correlation with the clinical response.

Correlation with patient response in clinic

Information about treatment responses were collected for all included patients. Subsequently, the corresponding cell lines were exposed to the same treatment (e.g. systemic agent or radiation). The clinical response was compared with the response of the corresponding cell line, to investigate if there was a correlation and if the cell lines could be used to predict treatment outcome. Clinical response was defined according to RECIST 1.1 (stable diseases (SD), partial response (PR) or complete response (CR) in clinic) at the first response evaluation, while response in the cell lines was defined as increased or decreased percentage of viable cells. A decrease of ≥50% of viable cells in the cell lines after 72 hours of exposure to the IC50 concentration of a systemic agent or nine days after exposure to 6 Gy of radiotherapy was defined as a concordant response to treatment.

Results

Patient recruitment and cell line establishment

Between March 2018 and February 2020, 32 patients were included (*Table 2*). In 22 patients (69%) 2D cell lines could successfully be established. The inclusion is still ongoing to further expand this platform of sarcoma cell lines. The included histologic sarcoma subtypes were four patients with myxoid liposarcoma, four patients with dedifferentiated liposarcoma, three patients with leiomyosarcoma, two patients with angiosarcoma, two patients with synovial sarcoma, two patients with undifferentiated pleomorphic sarcoma, two patients with sarcoma not otherwise specified (of which one patient turned out to be a malignant peripheral nerve sheath tumor after pathologic reassessment), one patient with chondrosarcoma, one patient with a malignant triton tumor and one patient with solitary fibrous tumor. Success rate of the first nine patients was low (33%), but after optimization of our culture methods the success rate of establishing 2D cell lines increased to 87%. Not all patients gave informed consent for an additional blood draw and therefore HS was available for sixteen out of 32 patients.

Fifteen out of sixteen (94%) cell lines cultured in HS grew out to stable 2D cell lines. The development of 3D cell lines was more difficult. Nine out of fourteen cell lines turned into organoids and were stored at -80 degrees for further experiments (*Figure 1A & 1B*). Of these 3D cell lines, which formed organoids, six were cultured in medium with HS (*Figure 1B*). Further experiments are needed to confirm the correlation between the organoids and the primary tumor. Furthermore, *Figure 1B* provides an overview of all the initiated cell lines and the success rate in establishing stable (passage 4 (P4)) cell lines for the different culture methods (HS vs FBS).

Table 2. Overview of tumor characteristics of the established cell lines. An overview is provided of the 22 successful established cell lines with the tumor characteristics, including information about the location where tumor sampling was performed and if the tumor sample was taken from a primary, recurrent or metastatic lesion. MPNST=Malignant peripheral nerve sheath tumor, RT=radiotherapy.

Cell line	Tumor characteristics	Primary, recurrence or metastasis	Location
ANG01	Primary angiosarcoma of the breast	Primary	Breast
ANG02	Radiation induced angiosarcoma of the breast	Primary	Breast
CHO01	Chondrosarcoma	Primary	Costa
LMS02	Leiomyosarcoma	Metastasis	Lungs
LMS05	Leiomyosarcoma	Metastasis	Lower back
LMS06	Leiomyosarcoma	Primary	Vena cava
LPS01	Dedifferentiated liposarcoma	Primary	Abdominal
LPS02	Dedifferentiated liposarcoma	Metastasis	Upper leg
LPS03	Dedifferentiated liposarcoma	Primary	Retroperitoneal
LPS04	Dedifferentiated liposarcoma	Primary	Chest
MLS01	Myxoid liposarcoma	Primary	Upper leg
MLS02	Myxoid liposarcoma	Primary	Upper leg
MLS03	Myxoid liposarcoma	Primary	Groin
MLS06	Myxoid liposarcoma	Primary	Upper leg
NOS01	Spindle cell sarcoma not otherwise specified	Primary	Lower leg
NOS04	Malignant peripheral nerve sheath tumor	Metastasis	Lungs
RHA01	Malignant triton tumor (pleomorphic rhabdomyosarcoma with MPNST component)	Recurrence	Sacral region back
SFT01	Solitary fibrous tumor	Primary	Upper leg
SYS01	Synovial sarcoma	Metastasis	Lymph node
SYS02	Synovial sarcoma	Primary	Ankle
UPS01	Undifferentiated pleomorphic sarcoma	Primary	Upper leg
UPS02	Undifferentiated pleomorphic sarcoma	Metastasis	Musculus psoas

Culture method 2D cell lines

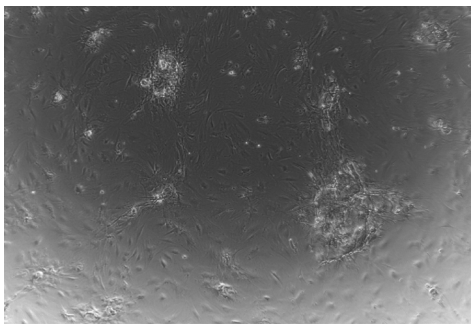
In sixteen cell lines we were able to compare the growth rate of the cell lines which were cultured in FBS containing medium with the cell lines cultured in HS containing

medium. The cell lines which were cultured in medium containing HS showed a growth advantage in eight out of sixteen (44%) cell lines, and a comparable growth rate in three (19%) cell lines, an inhibited growth rate in four (25%) cell lines, when compared to FBS, and failed in one (6%) cell line, with a median growth advantage of six days (*Figure 1C*).

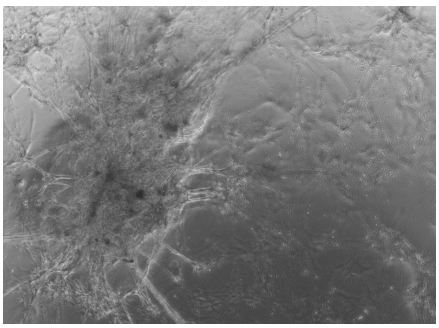
Figure 1. Cell culturing with FBS vs HS. ANG=angiosarcoma, CHO=chondrosarcoma, GIS=gastro intestinal stromal tumor, LMS=leiomyosarcoma, LPS=dedifferentiated liposarcoma, MLS=myxoid liposarcoma, NOS=sarcoma not otherwise specified, RHA= malignant triton tumor, SFT=solitary fibrous tumor, SYS=synovial sarcoma, UPS=undifferentiated pleomorphic sarcoma.

1A. Images 3D cell lines. On the left a photograph of the 3D cell line of angiosarcoma 1 cultured in fetal bovine serum was shown after passage 2. On the right the 3D cell line of myxoid liposarcoma 2 cultured in human derived serum after passage 5 was shown.

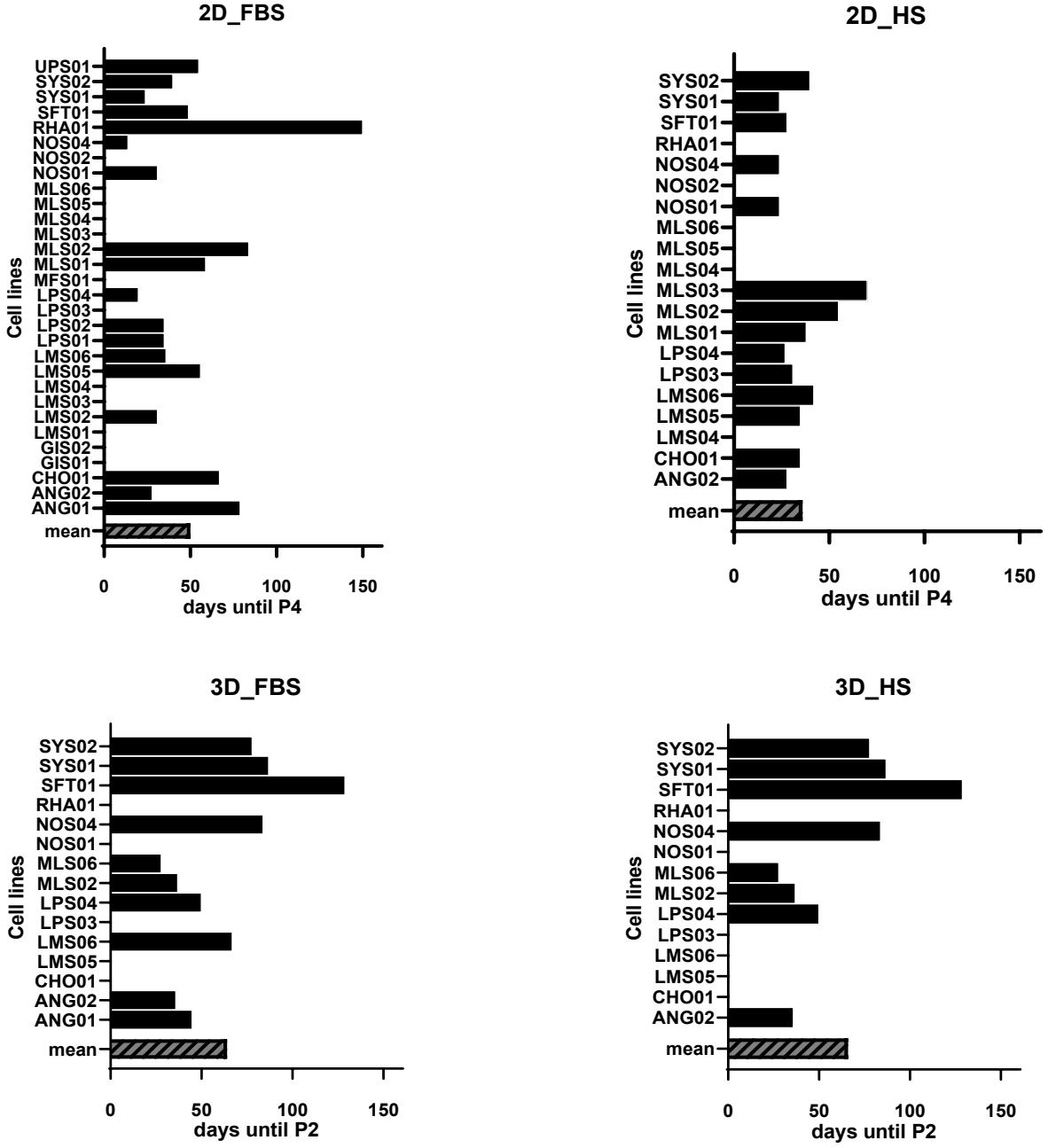
ANG01CC3, passage 2, cultured in FBS.



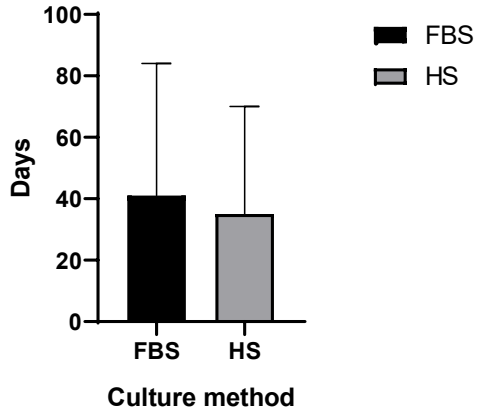
MLS02CC3, passage 5, cultured in HS.



1B. Cell lines cultured with FBS vs HS for the 2D and 3D cell lines. The upper graphs show the 2D cell lines, which were cultured with FBS (left) or HS (right). The lower graphs show the 3D cell lines, which were cultured with FBS (left) or HS (right). At passage 4 (P4)the cell lines were stated as stable cell lines and were partially stored frozen for future experiments.



1C. Growth rate of cell lines, FBS compared with HS. Figure 1 shows the median amount of days between the initiation of the cell lines, cultured with FBS- or HS-based medium, until the cell lines were at passage 4, with the upper limit. (N=16)



Viability assay systemic compounds

After a median culture period of seventeen days a viability assay could be initiated in the cell lines. Twelve regimens of systemic therapy were prescribed in eight patients (*Table 3*). The clinical response to systemic therapy could correctly be predicted in eight out of twelve cases (67%) for the FBS cultured cell lines and in five out of seven cases (71%) for the HS cultured cell lines (*Table 3*).

Viability assay radiotherapy

Eleven patients received radiotherapy (*Table 4*). A correlation was observed between the clinical response and the response in the cell lines in seven out of eleven cases (64%) for the cell lines cultured in FBS and in four out of five cases (80%) for the cell lines cultured in HS (*Table 4*).

Table 3. Correlation between clinical response and response of the cell lines after exposure to systemic therapy. The cell lines were exposed to the IC50 concentration of different systemic therapies. After 72 hours of incubation the percentage of viable cells was analyzed with a viability assay using cell titer blue. The amount of cells measured without any exposure to a systemic therapy was set to 100%. Responses were shown in green and no responses were shown in red.
ANG=angiosarcoma, LMS=leiomyosarcoma, NOS=sarcoma not otherwise specified, RHA=malignant triton tumor, SYS=synovial sarcoma, UPS=undifferentiated pleomorphic sarcoma.

Patient	**Treatment**	**Clinical response**	**Response of 2D culture** (% viable cells at IC50)	
			FBS	**HS**
ANG01	Paclitaxel	Mixed response	73%	-
ANG02	Doxorubicin + docetaxel	nCR	80%	11%
LMS02	Doxorubicin	SD	45%	-
	Trabectedin	SD	29%	
LMS05	Doxorubicin	SD	73%	57%
	Pazopanib	PD	102%	103%
NOS01	Melphalan	PD	77%	69%
	Doxorubicin	PD	81%	52%
	Ifosfamide	PD	49%	11%
RHA01	Doxorubicin	PD	66%	-
SYS01	Pazopanib	PD	92%	103%
UPS01	pazopanib	PD	100%	-

Table 4. Correlation between clinical response and response of the cell lines after exposure to radiotherapy. The cell lines were exposed to 6 Gy. After nine days of incubation the percentage of viable cells was analyzed with a viability assay using cell titer blue. The amount of cells measured after exposure to 0 Gy was set to 100%. Responses were shown in green, no responses were shown in red. ANG=angiosarcoma, LMS=leiomyosarcoma, LPS=liposarcoma, MLS=myxoid liposarcoma, NOS=sarcoma not otherwise specified, RHA=malignant triton tumor, SFT=solitary fibrous tumor, SYS=synovial sarcoma, UPS=undifferentiated pleomorphic sarcoma.

Patient	**Treatment**	**Clinical response**	**Response of 2D culture** (% viable cells after exposure to 6 Gy) **and correlation with the clinic**	
			FBS	**HS**
ANG02	RT	PD	11%	100%
LMS02	RT	SD	19%	-
LPS02	RT	SD	108%	-
MLS01	RT	PR	15%	22%
MLS02	RT	PR	27%	-
MLS03	RT	PR	155%	-
MLS06	RT	Response	94%	73%
RHA01	RT	PR	27%	-
SFT01	RT	SD	28%	22%
SYS01	RT	SD	19%	45%
UPS01	RT + pazopanib	SD	14%	-

Discussion

With this pilot study we have established a platform of 2D and 3D cell lines of sarcoma patients, which can be used for further investigations. In total, 22 sarcoma cell lines were established, which makes this one of the largest sets of published primary sarcoma cell lines (9,14–16,22) Even cell lines of the more rare histologic sarcoma subtypes were established, like angiosarcoma, malignant triton tumor and solitary fibrous tumor (8,12). In the beginning almost half of the biopsied tissues, did not attach or grow out in the well plates. A possible explanation was that most of the tumor cells in biopsied specimens of the failed cell lines were already apoptotic, due to longer time (>1 hour) between the biopsy and the start of the digestion step. Another explanation is that some pre-radiated tumors were also included, but these tumors were too apoptotic. After improving the logistic steps of the transport between the biopsy and the start of tumor digestion and exclusion of pre-radiated tumors, the success rate of establishing 2D cell lines improved from 69% to 87%. This is higher than reported success rates in literature, which ranged from 30-58% (9,14–16).

Despite the successful establishment of the 2D cell lines of the sarcoma patients in this study, the establishment of the 3D cell lines was more challenging. The take rate of the organoids was much lower than for the 2D cell lines, weeks instead of days, which made it not feasible to use the 3D cell cultures for the sensitivity assays. Furthermore, some of the organoids seemed to just disappear after a few passages. Addition of several growth factors and inhibitors to the medium could be necessary to obtain long term cultures, although other studies which culture organoids of other malignancies also use medium without the addition of growth factors. Also, adding many additives to the medium might result in long term culturing of the organoids, but it is questionable whether this will result in better response correlation in the clinical setting (20).

Culturing sarcoma cell lines in HS-containing medium did result in a growth advantage with a median of six days. This could aid in a timelier advice for the most suitable treatment for a patient. In this pilot study, a response prediction to a specific treatment could be provided within a month. Furthermore, the cell lines cultured in HS also seemed to predict the response to systemic and radiotherapy similar to even more accurately, when compared to FBS-cultured cell lines (71 and 80% compared to 67 and 64%, respectively). However, there are also downsides to this new culture strategy. The collection of 65 mL blood for this study could be a burden for patients, although when we would apply this technique in the clinic as a fast personalized drug and radiation sensitivity biomarker based predictive tool, only half of the blood volume will be necessary for just the sensitivity analysis.

Another advantage of this study was that the viability assays were performed with low passage cell lines (passage 4 to 8), which contain more heterogeneous cell populations with limited loss of specific genetic and biologic characteristics (17,18). This results in a more comparable situation with the primary tumor and, therefore, in a probably better correlation with the clinical response to treatment. Furthermore, this is one of the first studies in sarcoma, in which the response in cell lines to systemic treatment and radiotherapy was correlated to clinical responses of the same patients.

This is a promising first step to a fast personalized drug and radiation sensitivity biomarker for sarcoma patients. The current cutoff values of the responses seen in the cell lines were based on the best fit of correlation with clinical response. However, the best cutoff value for this predictive assay will probably be adjusted after further validation has been accomplished. Because of the heterogeneous nature of sarcoma, more patients per histologic subtype need to be included, to evaluate the precision of the cell lines as a fast personalized drug and radiation sensitivity biomarker for treatment prediction. Also, the cell lines need to be characterized first via sequencing or immunohistochemistry to prove their likeliness with the primary tumor.

Conclusion

HS can be used for culturing sarcoma cell lines instead of FBS. Clinical treatment response to systemic compounds could be predicted in 67% of the FBS cultured cell lines and in 71% of the HS cultured cell lines. This was 64% in the FBS cultured cell lines and 80% of the HS cultured cell lines for radiotherapy. Advantages of HS over FBS were the improved success rate of establishing stable cell lines (94% and 87% respectively), the enhanced growth rate with a median of six days and the slightly better correlation between the clinic and responses in the cell lines, which makes HS cultured cell lines more feasible in a predictive model for treatment response.

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Chapter 3
Enhancing anti-tumor response by combining checkpoint inhibitors with chemotherapy in solid tumors.

CHAPTER 3

Enhancing anti-tumor response by combining checkpoint inhibitors with chemotherapy in solid tumors.

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Published in Annals of Oncology 2019 Feb 1: 30(2):219-235.
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Abstract

Background: Cancer immunotherapy has changed the standard of care for a subgroup of patients with advanced disease. Immune checkpoint blockade (ICB) in particular has shown improved survival compared with previous standards of care for several tumor types. Although proven to be successful in more immunogenic tumors, ICB is still largely ineffective in patients with tumors that are not infiltrated by immune cells, the so-called cold tumors.

Patients and methods: This review describes the effects of different chemotherapeutic agents on the immune system and the potential value of these different types of chemotherapy as combination partners with ICB in patients with solid tumors. Both preclinical data and currently ongoing clinical trials were evaluated. In addition, we reviewed findings regarding different dosing schedules, including the effects of an induction phase and applying metronomic doses of chemotherapy.

Results: Combining ICB with other treatment modalities may lead to improved immunological conditions in the tumor microenvironment and could thereby enhance the antitumor immune response, even in tumor types that are so far unresponsive to ICB monotherapy. Chemotherapy, that was originally thought to be solely immunosuppressive, can exert immunomodulatory effects which may be beneficial in combination with immunotherapy. Each chemotherapeutic drug impacts the tumor microenvironment differently, and in order to determine the most suitable combination partners for ICB it is crucial to understand these mechanisms.

Conclusion: Preclinical studies demonstrate that the majority of chemotherapeutic drugs has been shown to exert immunostimulatory effects, either by inhibiting immunosuppressive cells and/or activating effector cells, or by increasing immunogenicity and increasing T-cell infiltration. However, for certain chemotherapeutic agents timing, dose and sequence of administration of chemotherapeutic agents and ICB is important. Further studies should focus on determining the optimal drug combinations, sequence effects and optimal concentration–time profiles in representative preclinical models.

Introduction

Drug development in oncology is shifting from targeting intrinsic properties of cancer cells to the tumor microenvironmental and the immune system of the host. Boosting T-cell memory may lead to more durable anticancer responses than seen with conventional anticancer therapy [1]. Endogenous anticancer response can be enhanced by blocking inhibitory checkpoint molecules. These checkpoint molecules function by dampening immune cells, a mechanism that prevents auto-immunity. Tumors utilize checkpoint inhibition in order to prevent T-cell-mediated tumor cell killing, by upregulating the ligands of checkpoint inhibitors, such as PD-L1. Activating checkpoint inhibition pathways turns T-cells anergic and leads to T-cell exhaustion.

Approved drugs for immune checkpoint blockade (ICB) include the anti-PD1 antibodies nivolumab and pembrolizumab, the anti-PD-L1 antibodies atezolizumab, avelumab and durvalumab, and the anti-CTLA-4 antibodies ipilimumab and tremelimumab. ICB has been approved for use in a wide range of tumors, including melanoma, non-small cell lung cancer (NSCLC), renal cell cancer, Merkel cell cancer, Hodgkin's lymphoma, urothelial cancer and mismatch repair deficient (dMMR)/microsatellite instability high (MSI-H) tumors [1, 2, 3, 4, 5].

Extensive research has been carried out identifying factors contributing to response to ICB (*Table 1*). Currently approved biomarkers for ICB are PD-L1 expression and dMMR/MSI-H tumor status [42, 43]. In practice, lactate dehydrogenase (LDH) and tumor mutational burden are also commonly used to select patients that are thought to benefit from ICB treatment [6, 8]. Other biomarkers that have been identified include but are not limited to, tumor-infiltrating lymphocytes (TILs), $CD8^+$ T-cells, T-cell receptor clonality and IFN-γ-related gene signatures [6, 9, 10, 21, 24, 33, 37].

It is thought that ICB has the highest likelihood of success in tumors that have an inflamed phenotype [44, 45]. These inflamed phenotypes typically have a tumor microenvironment with functional $CD8^+$ TILs, functional antigen presentation machinery proteins, and T-helper type 1 cytokines and chemokines such as IFN-γ and IL-2 [27, 29, 46]. While there is an active immune response, inhibitory factors may also be present. Potential inhibitory factors are large densities of Tregs, MDSCs, and anti-inflammatory T-helper type 2 cytokines, such as TGF-β and IL-10 [47].

Other immunological phenotypes that can be found in the tumor microenvironment include a phenotype which is completely deprived of immune cells (immune desert), or a phenotype in which the immune cells are unable to infiltrate the tumor properly (immune-excluded tumors) [46, 48, 49]. These tumors lack infiltration of competent T-cells, rarely express checkpoint inhibitor molecules, have a low mutational load, and have low expression of antigen presentation machinery markers [48]. These two phenotypes rarely respond to ICB monotherapy [19].

Table 1. Predictive factors for checkpoint inhibition therapy. ECOG = Eastern Cooperative Oncology Group. LDH = lactate dehydrogenase. MDSC = myeloid derived suppressor cell. NK cells = natural killer cells. NSCLC = non-small cell lung cancer. OS = overall survival. PFS = progression-free survival. TCR = T-cell receptor. TILs = tumor infiltrating lymphocytes. TNBC = triple negative breast cancer. Tregs = regulatory T-cells.

Type	Predictive factor	Effect	Cancer type	Checkpoint inhibitor	Reference
Clinical	Clinical condition	High ECOG Performance status is predictive for poor OS	Melanoma, NSCLC	Nivolumab, pembrolizumab, ipilimumab	Nakamura et al. 2016[8], Bagley et al. 2017[106]
	Clinical chemistry	High LDH is predictive of poor OS	Melanoma, TNBC	Nivolumab, ipilimumab	Nakamura et al. 2016[8], Nanda et al. 2016[9], Loi et al. 2017[10], Martens et al. 2016[15]
		High C-reactive protein is predictive of poor OS	Melanoma	Nivolumab, ipilimumab	Nakamura et al. 2016[8], Simeone et al. 2014[107]
		High levels of soluble CD73 is associated with poor OS and PFS	Melanoma	Nivolumab	Morello et al. 2017[108]
Tumor	Tumor mutational burden	High mutational load correlates with improved OR, durable clinical benefit, PFS and OS	Various	Pembrolizumab, nivolumab, ipilimumab, atezolizumab	Hugo et al. 2016[109], Rizvi et al. 2015[110], Snyder et al. 2014[111], Rosenberg et al. 2016[112]
	Mismatch repair status	Mismatch repair deficiency correlates with response	Any solid tumor with mismatch repair deficiency	Pembrolizumab	Le et al. 2015[113]
	Tumor PD-L1 expression	PD-L1 expression correlates with response	Various tumor types	Pembrolizumab, nivolumab, atezolizumab	Gettinger et al. 2016[114]; Herbst et al. 2014[24] Fuchs et al. 2018[115]
	Viral etiology	Human Papilloma Virus positivity correlates with response	Head and neck cancer	Pembrolizumab	Chow et al. 2016[12]
		Epstein-Bar virus positivity correlates with response	Gastric	Pembrolizumab	Kim et al. 2018[116]
Immunological		Baseline peritumoral and intratumoral PD-1 expression on	Melanoma	Pembrolizumab, nivolumab	Vilain et al. 2017[117],

	Tumor infiltrating lymphocytes	$CD8^+$ T-cells correlates with response and improved survival			
		High level of stromal TILs correlates with response	Melanoma, TNBC	Pembrolizumab	Loi et al. 2017[10], Tumeh et al. 2014[11]
		PD-L1 expression on TILs correlates with response	Various tumor types	Atezolizumab, pembrolizumab	Herbst et al. 2014[24],Dirix et al. 2018[118], Tumeh et al. 2014[11]
		High baseline FoxP3 and IDO expression correlates with clinical activity	Melanoma	Ipilimumab	Hamid et al. 2011[119]
		More clonal TCR repertoire correlates with response	Melanoma	Pembrolizumab	Tumeh et al. 2014[11]
	Peripheral blood	Low number baseline $Ki67^+EOMES^+CD8^+$ T-cells is associated with relapse	Melanoma	Ipilimumab	Wang et al. 2012[19]
		High percentage of baseline memory $CD45RO^+CD8^+$ T-cells correlates with improved survival	Melanoma	Ipilimumab	Tietze et al. 2017[47]
		Lower baseline level of peripheral NK cells correlates with improved survival	Melanoma	Ipilimumab	Tietze et al. 2017[18]
		High Neutrophil-to-Lymphocyte ratio is predictive of poor OS	NSCLC, melanoma	Nivolumab, ipilimumab	Bagley et al. 2017[106], Cassidy et al. 2017[45]
		Low absolute and relative lymphocyte count is predictive of poor OS	Melanoma	Nivolumab, ipilimumab	Nakamura et al. 2016[8], Simeone et al. 2014[107], Martens et al. 2016 [15]
		Low leukocyte count at baseline correlates with response	Melanoma	Ipilimumab	Gebhardt et al 2015[120]
		Low neutrophil count is associated with improved OS	Melanoma, NSCLC	Ipilimumab, nivolumab	Bagley et al. 2017[106], Ferrucci et al. 2016[121]
		Low baseline MDSCs correlates with improved OS	Melanoma	Ipilimumab	Kitano et al. 2014[14], Sade-Feldman et al. 2016[122]

		High levels of serum IFN- γ, IL-6, and IL-10 are associated with response	Melanoma	Nivolumab	Yamazaki et al. 2017[123]
		High frequencies of circulating Tregs is associated with improved OS	Melanoma	Ipilimumab	Martens et al. 2016[15]
		Low absolute monocyte count correlates with OS	Melanoma	Ipilimumab	Martens et al. 2016[15]
		High frequency of $CD14^{+}CD16^{-}$ $HLA\text{-}DR^{hi}$ monocytes correlates with response	Melanoma	Nivolumab, pembrolizumab	Krieg et al. 2018[124]
	Immune gene expression	Upregulated IFN-γ signaling correlates to better response rates and better PFS rates	Head and neck cancer, NSCLC, melanoma, urothelial cell cancer	Pembrolizumab, nivolumab, atezolizumab	Ribas et al. 2015[13],Ayers et al. 2017[125], O'Donnell et al. 2017[126], Prat et al. 2017[127] Fehrenbacher et al. 2016[128].
		Upregulated T helper type 1 gene expression at baseline correlates to response	Various	Atezolizumab	Herbst et al. 2014[24]
		Upregulated genes regarding antigen presentation machinery correlates to better response rates and better PFS rates	Head and neck cancer, NSCLC, melanoma, urothelial cell cancer	Pembrolizumab, nivolumab	O'Donnell et al. 2017[126], Prat et al. 2018[127]
		Upregulated genes regarding T-cell cytotoxic function correlates to better response rates and better PFS rates	Head and neck cancer, NSCLC, melanoma, urothelial cell cancer	Pembrolizumab, nivolumab	O'Donnell et al. 2017[126], Prat et al. 2019[127]

In order to convert these immune deserts or immune-excluded tumors into inflamed tumors, combination therapy with either other immunotherapies or different treatment modalities [50], including chemotherapy, may be an option [48].

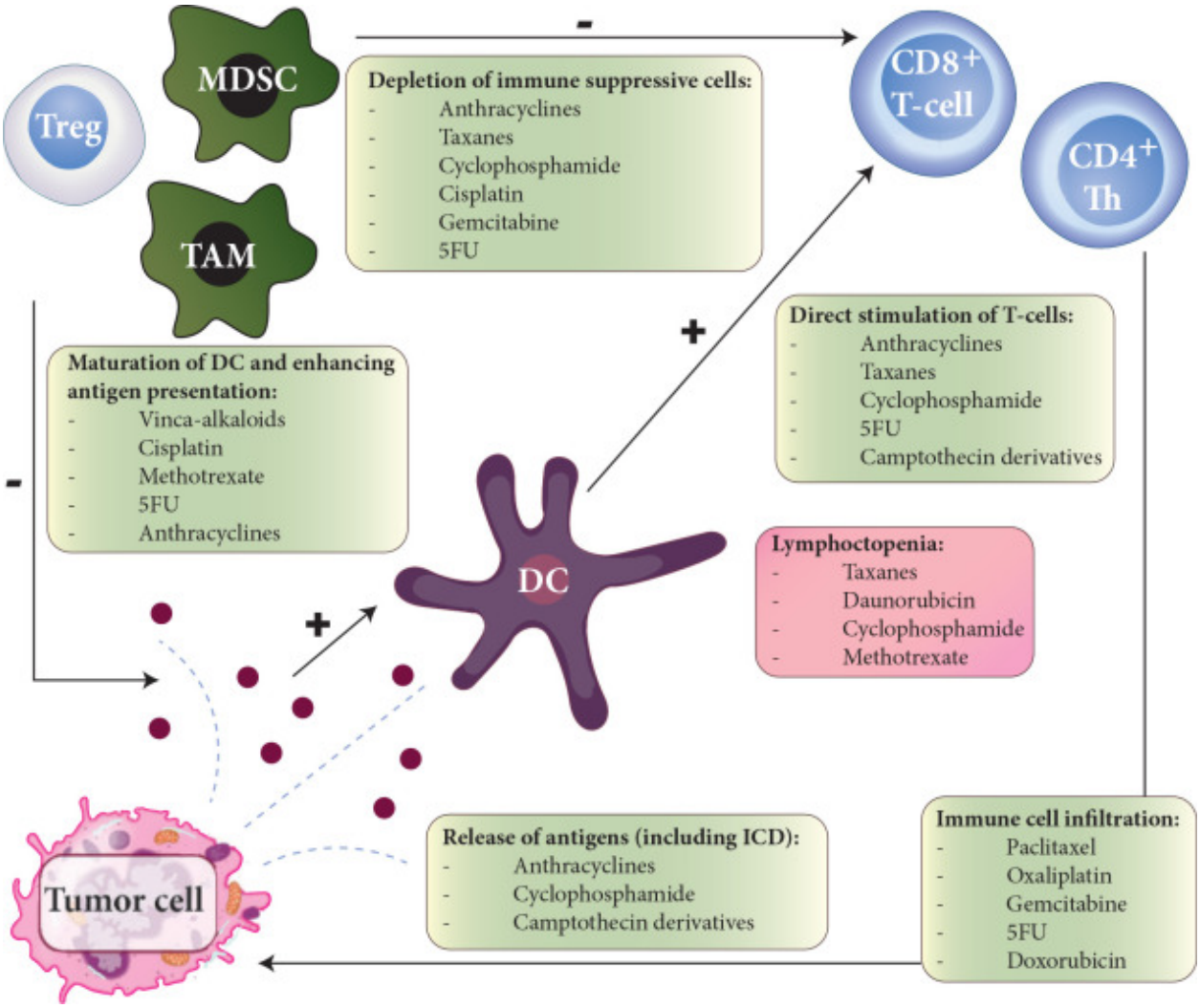
Chemotherapy was previously thought to be solely immunosuppressive, but recent data show that it may also possess immunostimulatory properties [51, 52]. It has the potential to induce favorable immunogenic conditions within the tumor microenvironment, which may be difficult to achieve by just targeting immune cells [51, 52]. In this review, we describe these immunomodulatory effects for different classes of chemotherapy. Each compound exerts unique immunological effects, which may be either beneficial or detrimental to treatment with ICB. Furthermore, this review discusses the compounds and treatment schedules in ongoing combination studies.

Immunomodulatory effects of chemotherapy

Chemotherapy comprises a large group of molecules which target proliferating cells. Although chemotherapy predominantly affects cancer cells, proliferating benign cells such as immune cells may also be affected. For this reason, it was long assumed that chemotherapy is merely immunosuppressive. Indeed, chemotherapy may lead to myelosuppression and leukocytopenia. However, recent findings demonstrate that many forms of chemotherapy also exhibit immunostimulatory effects. Here, we discuss the immunomodulatory effects of the four main groups of chemotherapy: topoisomerase inhibitors, antimicrotubule agents, alkylating agents and antimetabolites (*Figure 1* and *supplementary Table S1*, available at Annals of Oncology online).

We searched PubMed for preclinical and clinical trials published before 13 December 2018. Interim analysis and early-release publications of American Society of Clinical Oncology (ASCO) and European Society for Medical Oncology (ESMO) were also reviewed. Only articles in English were included. The search terms were ‘immune checkpoint inhibitors’, ‘anti-PD-(L)1’, ‘anti-CTLA-4’, and the names of the ICB available to date, ‘immunomodulation’ and the specific actors of the immune responses, ‘topoisomerase inhibitors’, ‘antimicrotubule agents’, ‘alkylating agents and antimetabolites’ and the specific agents per group. We only discuss chemotherapeutic compounds which are used for the treatment of solid tumors. Abstracts were reviewed and relevant articles were assessed in full.

Figure 1. Immunomodulatory effects of chemotherapy. DC, dendritic cells; 5FU, 5-fluorouracil; ICD, immunogenic cell death; MDSC, myeloid derived suppressor cell; TAM, tumor-associated macrophages; Th, T-helper cell; Tregs, regulatory T-cells.



Topoisomerase inhibitors

Topoisomerase inhibitors block the action of topoisomerases, enzymes controlling topological changes in DNA structures. Type I topoisomerases cut one strand of a DNA double helix, whereas type II topoisomerase cut both strands. Important topoisomerase inhibitors in the treatment of solid cancers of which immunomodulatory effects are described include topoisomerase I inhibiting camptothecin derivatives and topoisomerase II inhibiting anthracyclines.

Camptothecin derivatives

Irinotecan and topotecan are camptothecin derivatives commonly used in the treatment of a wide variety of solid tumors. Preclinical findings suggest that they may enhance T-cell recognition of tumor cells. In melanoma, they are capable of upregulating tumor-specific antigens. *In vitro* models demonstrated that treatment

with topoisomerase I inhibitors led to increased expression of the antigens melan-a/MART-1 and TP53INP1. Overexpression of these antigens led to improved recognition of tumor cells by T-cells, and subsequently increased T-cell-mediated killing of these tumor cells [53, 54]. Another *in vitro* experiment revealed upregulation of the danger-associated molecular patterns (DAMPs) high mobility group box 1 protein (HMGB1) and heat shock protein 70 (HSP70) after irinotecan treatment [55]. DAMPs have the potential to induce dendritic cell maturation leading to an inflammatory antitumor response. Tumor cells surviving topotecan treatment have upregulated major histocompatibility complex I (MHCI) and Fas expression, making them more sensitive to effector T-cell killing [56, 57].

Clinical studies determining the impact of camptothecin derivatives and individual drug doses and schedules on the immune system are limited in number. Small studies have been carried out monitoring changes in immune cell subsets in patients undergoing treatment. Camptothecin derivatives appear to impact the composition of immune cells in peripheral blood little, compared with other chemotherapeutic drugs. Topotecan treatment did not significantly impact absolute lymphocyte count nor T-cell and B-cell numbers in ovarian cancer patients with advanced disease [58]. However, the naive T-cell subpopulation was decreased upon treatment in chemotherapy naive patients, whereas the proportion of memory T-cells remained the same [58].

Anthracyclines

Anthracyclines are topoisomerase II inhibitors capable of inducing immunogenic cell death (ICD), a form of apoptosis which can induce an effective antitumor immune response through activation of DCs and the subsequent activation of specific T-cell responses. It is characterized by the expression of DAMPs, such as calreticulin, ATP, HMGB1 and HSP70 [59, 60]. *In vitro* studies demonstrated that DAMPs could be detected after 12 h of treatment and remained elevated through 24 h [61]. The dosage needed for induction of ICD, however, was generally higher than the dose needed for cytotoxicity [62]. ICD may also lead to the production of immunostimulatory cytokines, such as IFN-γ [62]. Inhibition of caspase, or depletion of DCs or $CD8^+$ T-cells may abolish anthracycline-mediated antitumor immune response [63]. Doxorubicin, epirubicin and idarubicin are all known to induce ICD [52].

Apart from ICD, other immunomodulatory effects of anthracyclines have been investigated as well. For instance, anthracyclines are able to elicit an immune response in a similar manner as induced by viral pathogens [64]. An in vivo experiment using fibrosarcomas in mice demonstrated that intratumoral doxorubicin increased levels of transcripts associated with viral infections, including IFN-stimulated genes, genes

involved in the recruitment and activation of leukocytes, and *Cd274* (encoding PD-L1). Anthracyclines have also been shown to selectively deplete immunosuppressive cells. Administration of 5 mg/kg doxorubicin intraperitoneally Q3W may lead to decreased MDSCs numbers *in vivo*, which in turn lead to increased numbers of $CD4^+$ and $CD8^+$ T-cells, as well as increased expression of IFN-γ, granzyme B and perforin [65]. Epirubicin impairs the function of Tregs by blocking the interaction between FoxP3 and the NF-κB subunit p65 *in vitro* [66]. This has resulted in blocking Treg-mediated suppression of $CD8^+$ T-cells.

The potential negative effects of anthracyclines on the immune system have been investigated in small studies. A single dose of epirubicin appeared to not significantly decrease blood lymphocyte numbers [67]. Daunorubicin has been shown to induce cell death in both resting and active peripheral blood lymphocytes after 20 h of incubation. This may be a potential negative effect for ICB combination [68]. Assessment of dose and schedule dependency of the aforementioned effects in cancer patients is warranted.

Antimicrotubule agents

Antimicrotubule agents exert neoplastic effects by disrupting microtubules. The most widely used antimicrotubule agents are the taxanes and vinca-alkaloids.

Taxanes

Docetaxel and paclitaxel are the most commonly used taxanes in the treatment of cancer. Taxanes are known for inducing leukocytopenia, depleting both lymphocytes and neutrophils, which has been described previously in a model [69]. Given as a 3-weekly standard of care, taxane-induced leukocytopenia typically starts 10 days after infusion and restores to baseline levels ~3 weeks after infusion. Neutrophils are depleted more than lymphocytes [69], thereby improving the neutrophil-to-lymphocyte ratio to a more favorable one for ICB treatment [30]. However, various types of lymphocyte subsets are depleted, including $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$ and $CD45RO^+$ cells [70]. As some of these cells are positively correlated with ICB response [28], further research is necessary to understand whether and how leukocytopenia affects ICB outcome.

Taxane treatment reduces the number of lymphocytes, but it is debatable whether they impact the functionality of cytotoxic T-cells. One study found that T-cell-mediated cytotoxicity was found to be impaired upon paclitaxel treatment [71]. In contrast, another study found no effect, [72], while others even found that taxane treatment led to increased NK and lymphokine activated killer cell activity [73].

Additionally, pro-inflammatory cytokines, such as IFN-γ, IL-2, IL-6 and GM-CSF were found to be upregulated after six cycles of standard taxane treatment [73].

Taxanes appear to selectively reduce immunosuppressive cells. Both docetaxel and paclitaxel have been shown to selectively decrease Treg and MDSC numbers, while unaffecting $CD4^+$ and $CD8^+$ viability [74., 75., 76., 77.]. Not only the number, but also the inhibitory function of Tregs is diminished. Expression of FoxP3, one of the key regulators of the immune system, was lowered in PBMCs which were incubated with paclitaxel for 24 h [77]. Another study found the anti-inflammatory cytokine IL-10 to be significantly decreased in patients with advanced disease after 4 weeks of paclitaxel treatment [72].

Taxane treatment may lead to induction of TILs [78]. A small prospective study showed that in breast cancer patients, tumors were non-inflamed before treatment. However, after four treatment cycles of 200 mg/m2 Q2W neoadjuvant paclitaxel treatment, surgery was carried out and one-third of the patients demonstrated immune infiltrates in their tumor biopsies. Interestingly, only the patients with a partial or complete response demonstrated TILs after treatment.

Vinca-alkaloids

Few studies have been carried out to assess the impact of vinca-alkaloids on the immune system. Vincristine suppresses the activity of immunosuppressive tumor-associated macrophages, whilst upregulating pro-inflammatory cytokines, and downregulating PD-L1 in PBMCs of healthy donors [79]. Vinorelbine generates reactive oxygen species and nitrogen species *in vivo*, which kills a significant number of immune cells [80], an effect which might negatively impact outcome of ICB. Vinblastine was identified as a compound capable of inducing maturation of DCs in an *in vitro* drug screen. Subsequent *in vivo* experiments revealed that administration of vinblastine enhanced $CD8^+$ clonal expansion and cytotoxic function [81]. Here, vinblastine was administered subcutaneously twice, with 1 week between injections.

Alkylating and platinum-based anticancer agents

Alkylating agents inhibit a.o. the transcription of DNA into RNA, thereby stopping protein synthesis.

We discuss the most widely used compounds of this class of drugs: cyclophosphamide, dacarbazine and platinum-based chemotherapeutic drugs.

Cyclophosphamide

Cyclophosphamide is extensively investigated for its immunomodulating effects. Similar to anthracyclines, cyclophosphamide is capable of inducing ICD [52].

Furthermore, cyclophosphamide may trigger DC homeostasis [82]. Mice which were injected with a single dose of intraperitoneal 100 mg/kg cyclophosphamide exhibited tumor cell death with immunogenic features, tumor infiltration and engulfment of apoptotic tumor cells by DCs, and subsequent cross priming of $CD8^+$ T-cells by DCs.

The dosage may be crucial when combining cyclophosphamide with immunotherapy. While higher dosages of cyclophosphamide induce myelosuppression [83], metronomic low dosing may boost the immune system [84., 85., 86.]. Patients receiving a daily dose of 100 mg oral cyclophosphamide showed decreased numbers and percentages of Treg cells, whereas there was no significant decrease in other lymphocyte counts [85]. Interestingly, doubling the dose of cyclophosphamide depleted all lymphocyte subpopulations. Next to decrease of Treg cells, effector functions are increased in patients receiving a metronomic dose. Both NK and T-cell activity were increased [85]. Furthermore, *in vivo* experiments show that a single low dose of cyclophosphamide leads to a shift from T-helper type 2 to type 1 cytokines, with enhanced IL-2 and IFN-γ production, and decreased IL-10 and TGF-β production after treatment compared with pretreatment [86]. A clinical trial investigating a modified vaccine Ankara-5T4 (MVA-5T4) further demonstrated the potential abilities of cyclophosphamide to deplete Treg cells. Patients were randomized to receive 50 mg twice daily cyclophosphamide or not, and MVA-5T4 or not [87]. In both cyclophosphamide group and the cyclophosphamide plus MVA-5T4 group, FoxP3 Treg cells were depleted. These depletions were noted at week three of treatment, which was associated with a longer progression-free survival (PFS). In the cyclophosphamide only group, Treg numbers returned to baseline at day 29. Although various studies illustrated positive effects of metronomic low dosing of cyclophosphamide, other studies showed no difference or even increase in Treg levels upon treatment [88, 89].

Next to the dosage and schedule, the tumor type may play a role as well in triggering a drug induced-immune response. Treg depletion was observed in breast cancer and CRC [87, 90], but not in melanoma patients [91]. Cyclophosphamide eliminated MDSCs in CRC [92], but in prostate cancer patients, metronomic cyclophosphamide treatment led to an increase of MDSC [91].

Dacarbazine

Dacarbazine is currently only used in melanoma patients for which the newer therapies are contra-indicated or who progressed on other therapies. Dacarbazine upregulates NKG2D receptors in human melanoma cells, which leads to activation of NK cells and release of IFN-γ. Increased levels of IFN-γ results in upregulation MHCI-expression on tumor cells, which is necessary for the recognition by T-cells [93].

Platinum derivatives

Well-known platinum derivatives are cisplatin, oxaliplatin and carboplatin. Cisplatin is the best studied platinum derivative regarding immunomodulatory effects. Cisplatin was shown to upregulate tumor cell expression of PD-L1, e.g. in head and neck squamous cell carcinoma patients receiving standard cisplatin treatment [94]. This suggests a post-exposure anticancer T-cell response, hampered by coinciding PD-L1 expression. High doses of cisplatin significantly reduced IFN-γ production by T-cells *in vitro* [95], and reduced the cytotoxicity of NK cells in ovarian cancer patients [96]. Lower doses impaired T-cell function less significantly [95]. Conversely, aside from the immunosuppressive effects through PD-L1 upregulation, cisplatin has been shown to have immunostimulatory properties as well, demonstrated by upregulation of MHC class I expression on antigen presenting cells [97, 98], recruitment of effector cells to the tumor site, triggering their proliferation [99], and downregulation of the immunosuppressive microenvironment by depleting MDSCs and Tregs [100].

Less data are available regarding the effect on the immune system of oxaliplatin and carboplatin. Oxaliplatin induces upregulation of PD-L1 on DCs [101], and carboplatin PD-1 mRNA expression [102]. Oxaliplatin may induce novel T-cell infiltration of the tumor. A single dose of oxaliplatin increased immune-cell infiltration in a CRC mouse model [103]. Furthermore, oxaliplatin is a known ICD inducer [104] and upregulates DAMPs [105].

Clearly, also of this class of oncolytics optimal dose and schedule for boosting ICD needs to be further established.

Antimetabolites

Antimetabolites interfere with essential biochemical pathways for DNA synthesis, often acting as a substitute or competitor of the natural substrates in physiological metabolism. We focused on the compounds that are often used in solid tumors: gemcitabine, methotrexate and 5-fluorouracil (5FU).

Gemcitabine

The immunomodulating properties of gemcitabine are mainly investigated when applied at the standard dose. Administration of this dose decreases the number of MDSCs, while enhancing cross-presentation of malignant antigens [106]. In pancreatic cancer patients, standard dose gemcitabine led to the depletion of Tregs, which lasted until 2 weeks after the last dose of chemotherapy [107]. Interestingly, no other lymphocyte subtypes significantly decreased after treatment. In ovarian cancer, a single dose of gemcitabine increased the $CD8^+$ T-cell tumor infiltration and PD-L1 expression both *in vitro* and *in vivo* [107, 108]. This effect was observed during the first 5 days after treatment, but not after 2 weeks of treatment [102]. Due to this time-

dependent effect, ICB could best be given 1 week after gemcitabine administration. The impact of dose on immunomodulatory effects require further investigation.

Methotrexate

Methotrexate targets rapidly dividing cells by inhibiting the formation of nucleotides, thereby impairing proliferation. Although high-dose methotrexate causes bone marrow suppression [109], low-dose methotrexate has shown immunostimulating properties. In an *in vitro* experiment, low-dose non-cytotoxic concentrations of methotrexate boosted the maturation of DCs by upregulating CD40, CD80 and CD83 [110]. In return, the DCs stimulated proliferation of T-cells [110], which could lead to a greater antitumor response. This suggests that low-dose methotrexate could be used as an immunostimulating agent. However, more research evaluating the impact of methotrexate on the immune system is needed to confirm whether it is indeed a suitable combination partner for ICB, as the currently available data are too limited.

5-Fluorouracil

5FU functions as antimetabolite of pyrimidine by inhibiting the synthesis of DNA and RNA. 5FU is the most extensively investigated oncolytic compound for its immunomodulating effects.

A standard dose of 5FU may exert immune stimulatory effects, e.g. by facilitating antigen uptake by DCs. In an *in vitro* experiment, DCs were incubated with a gastric cancer cell line which was pretreated with 5FU. The isolated DCs showed higher IL-12 production when incubated with the gastric cancer cell line pretreated with 5FU compared with the control. Subsequently, the cytotoxic T-lymphocytes generated by these DCs showed higher cytotoxicity compared with the control [111]. Furthermore, 5FU also selectively kills MDSCs *in vivo*, while sparing the other lymphocyte subtypes [108]. Effects were seen in the spleen and tumor of mice, 5 days after the intraperitoneal injection. Selective depletion of MDSCs was associated with greater $CD8^+$ T-cell tumor infiltration and T-cell-dependent antitumor responses.

Combination therapy

Various studies investigating combination therapy with chemotherapy and checkpoint inhibitors have been carried out. Both *in vivo* and clinical studies are showing promising results [103, 112, 113].

In vivo experiments allow for swift testing of different regimens by varying both the doses and the order of administration of the drugs. One study tested three different regimens using the combination of gemcitabine and ipilimumab in non-immunogenic mouse models [114]. Gemcitabine was given either 15 days before anti-

CTLA-4, concomitantly, or 3 days after anti-CTLA-4. Synergistic effects were only observed in the concomitant regimen, while omitting the first dose of gemcitabine drastically decreased antitumor effects. In another *in vivo* study combining cyclophosphamide and anti-CTLA-4 similar results were obtained [115]. Immunological antitumor responses were seen when cyclophosphamide was given 1 day before anti-CTLA-4 treatment. However, when reversing the order, $CD8^+$ T-cells underwent massive apoptosis and antitumor effects of anti-CTLA-4 were attenuated. These findings suggest that indeed there is a need for a chemotherapy induction phase before administering ICB.

An overview of clinical trials of which data are available is presented in Table 2. Combination therapy in the clinic is mostly well tolerated, and durable responses have been observed in various trials. Currently, three combinations have been approved for first-line treatment, all for advanced NSCLC [120, 147, 148].

In the majority of clinical trials, chemotherapy and ICB are administered concurrently and at full doses. Few trials have explored the optimal dose, or sequence of administration, while preclinical data have shown that these parameters might affect outcome. For example, an induction phase of chemotherapy can modify the tumor microenvironment thereby optimizing it for ICB [152]. A study in metastatic triple negative breast cancer (TNBC) patients investigated induction therapy with various types of chemotherapy [153, 154]. For the induction phase, low doses of chemotherapy were given for 2 weeks: 50 mg daily cyclophosphamide, twice 40 mg/m2 cisplatin or twice 15 mg doxorubicin. Response rates with chemotherapy appear higher in the cohorts where low-dose chemotherapy was used as induction, compared with nivolumab alone. Thus far, response rates appear most promising in the doxorubicin and cisplatin induction arms. Biomarker analysis carried out in this trial showed that indeed upon treatment with these two compounds, upregulation is found in key immunological pathways associated with response to anti-PD-1, and this effect is further increased after nivolumab administration. Furthermore, the number of intratumoral T-cells as well as the T-cell clonality is found to be higher after treatment with these drugs, compared with no induction phase [155]. Another study that investigates the impact of order of administration is a large phase II study of ipilimumab with paclitaxel and carboplatin in NSCLC patients [129]. Three different regimens were tested: a phased regimen in which chemotherapy is given before ipilimumab, a concurrent regimen, and a control group of placebo and chemotherapy. The primary end point of improved PFS was only met in the phased regimen, suggesting again that there is indeed a need for a chemotherapy induction phase.

Conversely, a potential immunotherapy induction phase may also be useful. This type of induction could prevent the adverse effects of chemotherapy on the immune system

and could improve the overall response rate of combination therapy [121]. A study in TNBC showed that an induction phase with durvalumab followed by combination therapy of weekly nab-paclitaxel for 12 weeks followed by four cycles of combination therapy with epirubicin and cyclophosphamide resulted into a higher pathological CR rate when compared with chemotherapy alone (53.4% versus 44.2%, respectively) [123]. As there was no chemotherapy induction arm in this trial, it remains to be elucidated whether an immunotherapy or chemotherapy induction phase is most effective.

Table 2. Clinical combination trials. AUC, area under the curve; b.i.d., twice daily; CI, confidence interval; CR, complete response; D, day; DCR, disease control rate; 5FU, 5-fluorouracil; ICB, immune checkpoint inhibition; MTD, maximum tolerated dose; NSCLC; non-small cell lung cancer; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; Q×W, every×weeks; rAE, related adverse events; STS, soft tissue sarcoma; TNBC, triple negative breast cancer.

Drugs Immunotherapy	Chemotherapy	Regimen	Tumor Type	Reference	Major findings/issues
Atezolizumab	Nab-paclitaxel	Atezolizumab 800 mg Q2W (d1,15) and nab-paclitaxel 125 mg/m^2 Q1W (d1,8,15) in cycles of 4 weeks	TNBC	Adams et al. 2018[129]	Median PFS 5.5 months, OS 14.7months
Atezolizumab	Carboplatin, paclitaxel, nab-paclitaxel	Atezolizumab 15mg/kg(in a later amendment 1200mg flat dose) + carboplatin AUC6 + paclitaxel 200mg/m^2 Q3W(Arm C), atezolizumab + carboplatin + pemetrexed 500mg/m^2 Q3W(Arm D), or atezolizumab + carboplatin Q3W + nab-paclitaxel 100mg/m^2 QW (Arm E) for 4–6 cycles followed with atezolizumab maintenance	NSCLC	Liu et al. 2018[130]	Confirmed ORRs were 36% Arm C, 68% Arm D (one complete response [CR]) and 46% Arm E (four CRs). Median PFS was 7.1 months, 8.4 months and 5.7 months, respectively. Median OS was 12.9 months, 18.9 months and 17.0 months, respectively
Atezolizumab	Carboplatin and etoposide	Carboplatin AUC5 + etoposide 100mg/m^2 Q3W + atezolizumab 1200mg or placebo Q3W	NSCLC	Horn et al. 2018[131]	The median OS was 12.3 months in the atezolizumab group and 10.3 months in the placebo group (hazard ratio for death, 0.70; 95% CI, 0.54 to 0.91; P=0.007). The median PFS was 5.2 months and 4.3 months, respectively (hazard ratio for disease progression or death, 0.77; 95% CI, 0.62 to 0.96; P=0.02).
Atezolizumab, durvalumab, nivolumab or pembrolizumab	Carboplatin, pemetrexed, bevacizumab, docetaxel, ramucirumab, vinorelbine,	Any ICB before any chemotherapy	NSCLC	Grigg et al.2017[132]	Durable responses after treatment. Lack of control arm

	gemcitabine, paclitaxel				
Atezolizumab	Carboplatin, paclitaxel, bevacizumab	Atezolizumab 1200 mg + Carboplatin AUC 6 + Paclitaxel 200 mg/m^2 (Arm A) or atezolizumab + bevacizumab 15 mg/kg + Carboplatin + Paclitaxel (Arm B) vs bevacizumab + carboplatin + paclitaxel (Arm C) IV q3w for 4 or 6 cycles per investigator discretion, then maintenance atezolizumab, atezolizumab + bevacizumab or bevacizumab, respectively.	NSCLC	Reck et al. 2017[94]	Clinically meaningful PFS benefit with atezolizumab + bevacizumab + chemotherapy versus bevacizumab + chemotherapy. Approved for first line treatment of metastatic non-squamous NSCLC.
Avelumab	Carboplatin, gemcitabine	2 cycles of induction avelumab prior to combining carboplatin-gemcitabine plus avelumab for 6 cycles	Urothelial cell cancer	Vida et al. 2018[101]	Induction phase with ICB. "Priming the immune response before chemotherapy could prevent the detrimental effect of chemotherapy on immune cells; reduction of tumor burden with chemotherapy may allow immunotherapy to be more effective"
Avelumab	Cisplatin	Avelumab 10mg/kg on Day 1 of the Lead-in Phase; Days 8, 25, and 39 of the chemoradiation therapy phase; and q2w for 12 months during the maintenance phase. •Cisplatin 100 mg/m^2 IV: Days 1, 22, and 43 of the chemoradiation therapy phase. Radiation therapy 70 Gy/ 35 fractions/ 7 weeks.	Head and neck cancer	Yu et al. 2018[133]	No results yet
Durvalumab	Nab-paclitaxel, cyclophosphamide, epirubicin	Durvalumab 1.5 g or placebo Q4W. placebo monotherapy (0.75 g) was given for the first 2 wks (window phase), and durvalumab/placebo + nab-paclitaxel 125 mg/m^2 QW for 12 wks, followed by durvalumab/placebo + epirubicin/cyclophosphamide Q2W for 4 cycles	TNBC	Loibl et al. 2018[134]	Combination therapy resulted in a high CR rate in TNBC. Induction therapy with durvalumab seemed beneficial

Durvalumab	Eribulin	A fixed dose of durvalumab (1.12g) is given on day 1 of each cycle. The starting dose is 1.1mg/m^2 with dose escalation to 1.4mg/m^2 on day 1 and day 8 Q3W	TNBC	Landry et al. 2018[135]	No results yet
ICB	Carboplatin, paclitaxel, temozolomide, nab-paclitaxel	Not specified in abstract/poster	Melanoma	Aguilera et al. 2018[136]	Patients who received chemo-immunotherapy had a median OS of 5 years (95% CI: 2-NR) versus 1.8 years (95% CI: 0.9-2; p = 0.002) for those who received either ICB (n = 9) or chemotherapy alone (n = 15), with ORR of 61% versus 17% (p = 0.001), respectively
Ipilimumab	Dacarbazine	Ipilimumab 10mg/kg + dacarbazine 850mg/m^2 or dacarbazine + placebo Q3W followed by dacarbazine monotherapy	Melanoma	Robert et al. 2011[137]	Combination therapy resulted into a higher OS (11.2 months vs. 9.1 months). In another phase II study ipilimumab monotherapy resulted into an OS of 11.1 month, even in pre-treated patients[138]
Ipilimumab	Dacarbazine	Ipilimumab 3mg/kg Q4W alone or with dacarbazine 250mg/m^2/day up to 6x5day cycles	Melanoma	Hersh et al, 2011[139]	Objective response rate was 14.3% vs 5.4% for the combination therapy. OS was 20.9 and 16.4 respectively
Ipilimumab	Paclitaxel, carboplatin	Concurrent: 4x ipilimumab 10mg/kg + paclitaxel 175mg/m^2 + carboplatin AUC6 followed by 2x placebo + paclitaxel + carboplatin Phased: 2x placebo + paclitaxel + carboplatin followed by 4x ipilimumab + paclitaxel + carboplatin. Control: placebo + paclitaxel + carboplatin	NSCLC	Lynch et al. 2012[100]	Only phased regimen leads to improved PFS compared to control
Ipilimumab	Gemcitabine, cisplatin	2x gemcitabine 1000mg/m^2 + cisplatin 70mg/m^2 followed by 4x ipilimumab 10mg/kg + gemcitabine + cisplatin	Urothelial cell carcinoma	Galsky et al. 2017[140]	No changes in composition and frequency of peripheral immune cells after gemcitabine. Expansion of CD4$^+$ cells after

					combination therapy. No ipilimumab monotherapy cohort
Ipilimumab	Paclitaxel, carboplatin	Phased ipilimumab paclitaxel + carboplatin followed by 4x 10mg/kg ipilimumab + paclitaxel + carboplatin	NSCLC	Govindan et al. 2017[141]	No prolonged OS compared to chemotherapy alone
Ipilimumab, nivolumab	Trabectedin	Ipilimumab (1mg/kg Q12W), nivolumab (3mg/kg Q2W), and escalating doses of trabectedin (1.0, 1.3, 1.5 mg/m^2 Q3W)	STS	Gordon et al. 2018[142]	No results yet
Nivolumab	Gemcitabine, cisplatin, pemetrexed, paclitaxel, carboplatin, docetaxel	Arm A: nivolumab 10mg/kg + gemcitabine 1250mg/m^2 + cisplatin 80mg/m^2 Arm B: nivolumab + pemetrexed 500mg/m^2 + cisplatin 75mg/m^2 Arm C: nivolumab + paclitaxel 200mg/m^2 + carboplatin AUC6 + bevacizumab 15mg/kg Arm D: nivolumab + docetaxel 75mg/m^2	NSCLC	Kanda et al. 2016[143]	Full dose was given. Difficult to compare response rates: different lines of treatment, different and low patient numbers
Nivolumab	Gemcitabine-cisplatin, pemetrexed-cisplatin, paclitaxel-carboplatin	10 mg/kg nivolumab gemcitabine-cisplatin, 10 mg/kg nivolumab pemetrexed-cisplatin, 10 mg/kg nivolumab paclitaxel-carboplatin 5 mg/kg nivolumab paclitaxel-carboplatin	NSCLC	Rizvi et al 2016[144]	Chemotherapy doses not defined. Most promising results in nivolumab- paclitaxel-carboplatin group
Nivolumab	Carboplatin	Carboplatin (AUC6) with or without nivolumab (360 mg) Q3W	TNBC	Garrido et al. 2018[145]	No results yet
Nivolumab	Paclitaxel	Nivolumab (Q4W, 3mg/kg on days 1 and 15 for level 1 and 1mg/kg for level −1) with fixed doses of paclitaxel and ramucirumab (Q4W, 80mg/m^2 on days 1,8 and 15 and 8mg/kg on days 1 and 15, respectively)	Gastric cancer	Nishina et al. 2018[146]	No results yet
Nivolumab	Paclitaxel	Nivolumab 240mg/body on day 1, 15, paclitaxel 90mg/m^2 on day1, 8, 15, and bevacizumab 10mg/kg on day 1, 15 Q4W	HER2 negative breast cancer	Ozaki et al. 2018[147]	No results yet

Nivolumab	Cisplatin, pemetrexed or cisplatin, gemcitabine	Cisplatin 75mg/m^2 Q3Wx3 plus either pemetrexed 500 mg/m^2 Q3Wx3 or gemcitabine 1250mg/m^2 d1, d8 Q3Wx3 plus nivolumab 360mg Q3Wx3	NSCLC	Evans et al. 2018[148]	No results yet
Nivolumab	Trabectedin	Trabectedin (1.5mg/m^2) Q3W, and nivolumab (3mg/kg) Q3W	STS	Chawla et al. 2018[149]	Paired administration of trabectedin and nivolumab is safe, and that this combined chemo-/immuno-therapy approach may have synergistic activity
Nivolumab	Cisplatin, pemetrexed	Cisplatin (75 mg/m^2), pemetrexed (500 mg/m^2), and nivolumab (360 mg) q3w.	Mesothelioma	Fujimoto et al. 2018[150]	No results yet
Pembrolizumab	Paclitaxel, doxorubicin, cyclophosphamide, carboplatin	Cohort A: pembrolizumab 200mg Q3W + nab-paclitaxel 100-125mg/m^2 QW followed by Q3W pembrolizumab 200mg + cyclophosphamide 600mg/m^2 + doxorubicin 60mg/m^2. Cohort B: pembrolizumab 200mg Q3W+ nab-paclitaxel 100-125mg/m^2 QW + carboplatin AUC6 Q3W followed by Q3W pembrolizumab 200mg + doxorubicin 60mg/m^2 + cyclophosphamide 600mg/m^2	TNBC	Bhatti et al. 2017[151]	Both regimens showed promising anti-tumor activity with manageable toxicity. Addition of carboplatin resulted in more grade 3 or 4 toxicities, mainly neutropenia
Pembrolizumab	Gemcitabine, docetaxel, nab-paclitaxel, vinorelbine, irinotecan, liposomal doxorubicin	Arm 1: pembrolizumab 2mg/kg Q3W+ gemcitabine 1000mg/m^2 D1 and D8 Q3W Arm 2: pembrolizumab + gemcitabine 900mg/m^2 D1 and D8 + docetaxel 75mg/m^2 D8 Q3W Arm 3: pembrolizumab + gemcitabine 1000mg/m^2+ nab-paclitaxel 125mg/m^2 D1 and D8 Q3W Arm 4: pembrolizumab + gemcitabine 1000mg/m^2+ vinorelbine 25mg/m^2 D1 and D8 Q3W Arm 5: pembrolizumab + irinotecan 300mg/m^2 Q3W Arm 6: pembrolizumab + liposomal doxorubicin 30mg/m^2 Q3W	All solid tumor types	Weiss et al. 2017[152]	All advanced solid tumor types included. Full doses of chemotherapy are used. Recommended phase 2 dose determined as maximum tolerated dose. Partial responses observed in arm 3 – 6.

Pembrolizumab	Carboplatin, pemetrexed,	Carboplatin AUC5mg/mL + pemetrexed 500mg/m^2 Q3W for 4 cycles followed by optional pemetrexed 500mg/m^2 +/- pembrolizumab 200mg Q3W for 2 years	NSCLC	Papadimitrakopoulou et al. 2017[90]; Langer et al.2016[153]	All drugs administered on same day. Improved efficacy over chemotherapy alone. Approved for first line treatment of metastatic non-squamous NSCLC.
Pembrolizumab	5FU, cisplatin	Pembrolizumab 200mg Q3W + cisplatin 80mg/m^2 Q3W + 5FU 800mg/m^2 Q3W or capecitabine 1000mg/m^2 BID Q3W	Gastric cancer	Bang et al.2017[115]	Full dose given. Promising anti-tumor activity irrespective of PD-L1 expression.
Pembrolizumab	Carboplatin, gemcitabine	Pembrolizumab 200mg Q3W, and carboplatin (AUC2) + gemcitabine (800mg/m^2) on days 1 and 8	TNBC	Obeid et al 2017[154]	Two out of three patients showed effective immune stimulation
Pembrolizumab	Eribulin	Eribulin 1.4mg/m^2 on day 1 and 8, pembrolizumab Q3W	TNBC	Tolaney et al 2017[155]	Median PFS 4.2 months, OS 17.7 months
Pembrolizumab	Capecitabine or paclitaxel	Pembrolizumab 200mg Q3W and 1st or 2nd line paclitaxel (80mg/m^2 qW) or oral capecitabine (2000mg BID, weekly 1 on/1 off)	TNBC	Page et al. 2018[156]	Three out of nine patients showed a partial response, of whom two had metastatic disease.
Pembrolizumab	Pemetrexed, cisplatin, carboplatin	Pemetrexed and a platinum-based drug +200mg pembrolizumab or placebo Q3W for 4 cycles followed by 35 cycles of pembrolizumab or placebo + pemetrexed	NSCLC	Gandhi et al. 2018[93]	OS at 12 months was 69.2% in the pembrolizumab-combination group versus 49.4% in the placebo-combination group, regardless of PD-L1 status. PFS survival was 8.8 vs 4.9 months respectively. The incidence of grade 3 rAEs was comparable between the 2 groups.
Pembrolizumab	Carboplatin, nab-paclitaxel	Pembrolizumab at 200mg/week plus carboplatin AUC6 Q3W and paclitaxel at 200mg/ m^2 Q3W or nanoparticle albumin-bound (nab)-paclitaxel at 100mg/ m^2 QW for 4 cycles vs the same chemotherapy plus placebo	NSCLC	Paz-Ares et al 2018.[157][95]	Improved overall survival (15.9 months vs 11.3 months), response rates, and duration of response (PFS if 6.4 months vs 4.8 months) in the group with chemo-immunotherapy compared to chemotherapy alone. Approved for first line

					treatment of metastatic squamous NSCLC.
Pembrolizumab	Docetaxel or gemcitabine	Pembrolizumab 200mg and either docetaxel 75 mg/m^2 (arm A) or gemcitabine 1000 mg/m^2 on day 1 and 8 (arm B) q3w	Urothelial cell cancer	Parikh et al. 2018[158]	Arm A had an ORR of 50% and DCR of 67%, whereas arm B had an ORR of 33% and DCR of 50%. Median PFS was 4.8, 5.7, and 3.7 months for the overall cohort, arm A, and arm B, respectively.
Tremelimumab	Gemcitabine	Gemcitabine (1000 mg/m^2 on days 1, 8, and 15 of each 28-day cycles) + tremelimumab (6, 10, or 15 mg/kg) on day 1 of each 84-day cycle for a maximum of 4 cycles	Pancreatic cancer	Aglietta et al. 2014[159]	Full dose gemcitabine, MTD of tremelimumab 15mg/kg. Two partial responses

Discussion

Conclusions and future perspectives

Checkpoint blockade therapy is effective in a variety of tumor types. However, to further increase the number of suitable tumor types, ICB may be combined with compounds which are able to convert non-inflamed tumors into inflamed ones. This in return may render these tumors more sensitive to ICB therapy. Preclinical studies demonstrate that the majority of chemotherapeutic drugs has been shown to exert immunostimulatory effects, either by inhibiting immunosuppressive cells and/or activating effector

cells, or by increasing immunogenicity and increasing T-cell infiltration. Although preclinical data have proved to be useful for identifying immunomodulating effects, extrapolation to the clinic should be done cautiously. For example, drug concentrations used in these experiments and drug exposure over time often do not correspond to observed exposure in the clinic. Preferably, preclinical experiments should mimic as much as is possible the clinical situation. An additional potential confounder is that the majority of studies investigate the immunomodulating effects of chemotherapy in peripheral blood and not in the tumor microenvironment. Although some peripheral factors contribute to a response to ICB, intratumoral immunological factors such as CD8+ T-cell infiltration, PD-L1 expression and IFN-γ secretion may be even more crucial and representative of observed effects. Therefore, it is warranted to further investigate the impact of chemotherapy in the tumor micro-environment. For this, it will be essential to draw pre- and on-treatment tumor biopsies during clinical trials, as they may reflect changes in the immunological status of the tumor better than peripheral markers.

In addition to choosing the ideal drugs for combination, it is crucial to investigate the optimal regimen for combination treatment. Current practice is that full-dose chemotherapy is administered with ICB on the same day. However, preclinical research suggests that for certain chemotherapeutic agents timing and sequence of administration of both modalities is important. Furthermore, during combination treatment, chemotherapy is now often administered at the maximum tolerated dose. For the majority of chemotherapeutic compounds, treatment at these doses results in bone marrow toxicity and may lead to altered immune cell function, while metronomic doses have been shown to augment immunotherapeutic activity [85, 156, 157]. Early signs of improved outcome of combined modality of chemotherapy and ICB in patients encourage more advanced approaches in identifying representative preclinical models, optimal drug combinations, sequence effects and ideal concentration–time profiles. This outcome should be the template for translation to clinical proof of concept studies, which should incorporate extensive pre- and on-

treatment biomarker assessment, which may leverage pivotal studies, ultimately leading to novel standards of care.

Funding
None declared.

Disclosure
JHB and JHMS are shareholder and part-time employee of Modra Pharmaceuticals bv. All remaining authors have declared no conflicts of interest.

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Chapter 4

Novel combinations of immunotherapy.

CHAPTER 4.1

Safety, Tolerability, and Potential Clinical Activity of a Glucocorticoid-Induced TNF Receptor–Related Protein Agonist Alone or in Combination With Nivolumab for Patients With Advanced Solid Tumors. A Phase 1/2a Dose-Escalation and Cohort-Expansion Clinical Trial

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Published in JAMA Oncology 2019;6(1):1-8. doi:10.1001/jamaoncol.2019.3848. Original Investigation.

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Abstract

Importance: Multiple immunostimulatory agonist antibodies have been clinically tested in solid tumors to evaluate the role of targeting glucocorticoid-induced tumor necrosis factor (TNF) receptor-related protein in anticancer treatments.

Objective: To evaluate the safety and activity of the fully human glucocorticoid-induced TNF receptor-related protein agonist IgG1 monoclonal antibody BMS-986156 with or without nivolumab in patients with advanced solid tumors.

Design, Setting, and Participants: This global, open-label, phase 1/2a study of BMS-986156 with or without nivolumab enrolled 292 patients 18 years or older with advanced solid tumors and an Eastern Cooperative Oncology Group performance status of 1 or less. Prior checkpoint inhibitor therapy was allowed. Monotherapy and combination dose-escalation cohorts ran concurrently to guide expansion doses beginning October 16, 2015; the study is ongoing.

Interventions: The protein agonist BMS-986156 was administered intravenously at a dose of 10, 30, 100, 240, or 800 mg every 2 weeks as monotherapy, and in the combination group 30, 100, 240, or 800 mg plus 240 mg of nivolumab every 2 weeks; same-dose cohorts were pooled for analysis. One cohort also received 480 mg of BMS-986156 plus 480 mg of nivolumab every 4 weeks.

Main Outcomes and Measures: The primary end points were safety, tolerability, and dose-limiting toxic effects. Additional end points included antitumor activity per Response Evaluation Criteria in Solid Tumors, version 1.1, and exploratory biomarker analyses.

Results: With a follow-up range of 1.4 to 101.7 weeks (follow-up ongoing), 34 patients (16 women and 18 men; median age, 56.6 years [range, 28-75 years]) received monotherapy (4 patients completed initial treatment), and 258 patients (140 women and 118 men; median age, 60 years [range, 21-87 years]) received combination therapy (65 patients completed initial treatment). No grade 3 to 5 treatment-related adverse events occurred with BMS-986156 monotherapy; grade 3 to 4 treatment-related adverse events occurred in 24 patients (9.3%) receiving BMS-986156 plus nivolumab, with no grade 5 treatment-related adverse events. One dose-limiting toxic effect (grade 4 elevated creatine phosphokinase levels) occurred in a patient receiving 800 mg of BMS-986156 plus 240 mg of nivolumab every 2 weeks; BMS-986156 with or without nivolumab exhibited linear pharmacokinetics with dose-related increase after a single dose. Peripheral T-cell and natural killer-cell proliferation increased after administration of BMS-986156 with or without nivolumab. No consistent and significant modulation of intratumoral $CD8^+$ T-cells and $FoxP3^+$ regulatory T-cells was observed. No responses were seen with BMS-986156 alone; objective response rates ranged from 0% to 11.1% (1 of 9) across combination therapy cohorts, with a few

responses observed in patients previously treated with anti-programmed death receptor (ligand) 1 therapy.
Conclusions and Relevance: Based on this cohort, BMS-986156 appears to have had a manageable safety profile, and BMS-986156 plus nivolumab demonstrated safety and efficacy comparable to historical data reported for nivolumab monotherapy.

Trial Registration: ClinicalTrials.gov identifier: NCT02598960.

Key Points

Question
Is the glucocorticoid-induced tumor necrosis factor receptor–related protein agonist BMS-986156 treatment with or without nivolumab tolerable and clinically active in patients with advanced solid tumors?

Findings
In this open-label, phase 1/2a study of 292 treated patients with advanced solid tumors (69 completed initial treatment), BMS-986156 therapy had a tolerable safety profile; combination therapy had a similar safety profile to that of nivolumab. No responses were seen with monotherapy; however, in combination therapy, response rates were comparable to those historically observed with nivolumab (<15% across tumor types).

Meaning
This study represents the largest data set on glucocorticoid-induced tumor necrosis factor receptor–related protein agonism with or without nivolumab to our knowledge; a clear signal has not emerged demonstrating that glucocorticoid-induced tumor necrosis factor receptor–related protein agonism may be an effective therapeutic strategy in a broad patient population.

Introduction

Immunotherapy treatment options are broad and include multiple approaches. One of the most successful strategies thus far has been the blockade of T-cell inhibitory checkpoints, such as cytotoxic T-lymphocyte–associated antigen 4 and programmed death receptor 1 (PD-1), and programmed death ligand 1 (PD-L1), to enhance the antitumor immune response.[1] Immune checkpoint inhibitors lead to highly durable responses and significantly prolonged overall survival in many advanced tumor types.[2-4] Dual checkpoint blockade, combining nivolumab with ipilimumab, has shown even more promising results in various tumor types.[5-10] Although checkpoint inhibitors have provided advancements in anticancer treatment, a significant majority of patients, such as those with tumor types that are mismatch repair proficient or noninflamed, remain unresponsive to checkpoint inhibition.[11-14] In addition, some patients with initial response to checkpoint inhibition eventually experience disease progression and face limited subsequent therapeutic options. Thus, new strategies for modulating the critical balance of T-cell activation and antigen tolerance that make an antitumor immune response possible are being investigated.[15-18]

A variety of T-cell costimulatory receptors exist whose activity and engagement may potentiate the T-cell response induced by checkpoint inhibitors. Promising therapeutic targets include the tumor necrosis factor receptor (TNFR) superfamily, such as the glucocorticoid-induced TNFR-related protein (GITR), TNFR superfamily member 4 (CD134/OX40), and TNFR superfamily member 9 (CD137/4-1BB). It was hypothesized that agonistic GITR antibodies may successfully activate costimulatory pathways to synergize with PD-1 inhibitors and PD-L1 inhibitors in the tumor microenvironment.[1] To date, several anti-GITR antibodies and anti-GITR ligand antibodies have been used in clinical trials.[19-26]

The protein agonist BMS-986156 is an IgG1 agonist monoclonal antibody to GITR, a molecule that is constitutively expressed by intratumoral regulatory T-cells at high levels and by effector T-cells at low levels.[27] In addition, GITR becomes upregulated on effector T-cells on their activation. In preclinical studies, GITR engagement can deplete GITR-expressing cells or can induce T-cell proliferation depending on the system.[27-28] BMS-986156 was engineered to increase T-cell activation and deplete intratumoral regulatory T-cells in combination with anti–PD-1 therapy. Preclinical experiments with in vivo syngeneic mouse models show enhanced antitumor activity when a GITR agonist antibody is added to anti–PD-1 therapy.[29] Thus, it was hypothesized that this combination may result in improved and prolonged antitumor responses in patients with advanced cancer.

In the present study, results are reported from a phase 1/2a dose-escalation and dose-expansion study investigating the safety, tolerability, pharmacokinetics,

pharmacodynamics, and preliminary clinical activity profiles of BMS-986156 as monotherapy treatment and in combination with nivolumab in patients with advanced solid tumors (NCT02598960).[30]

Methods

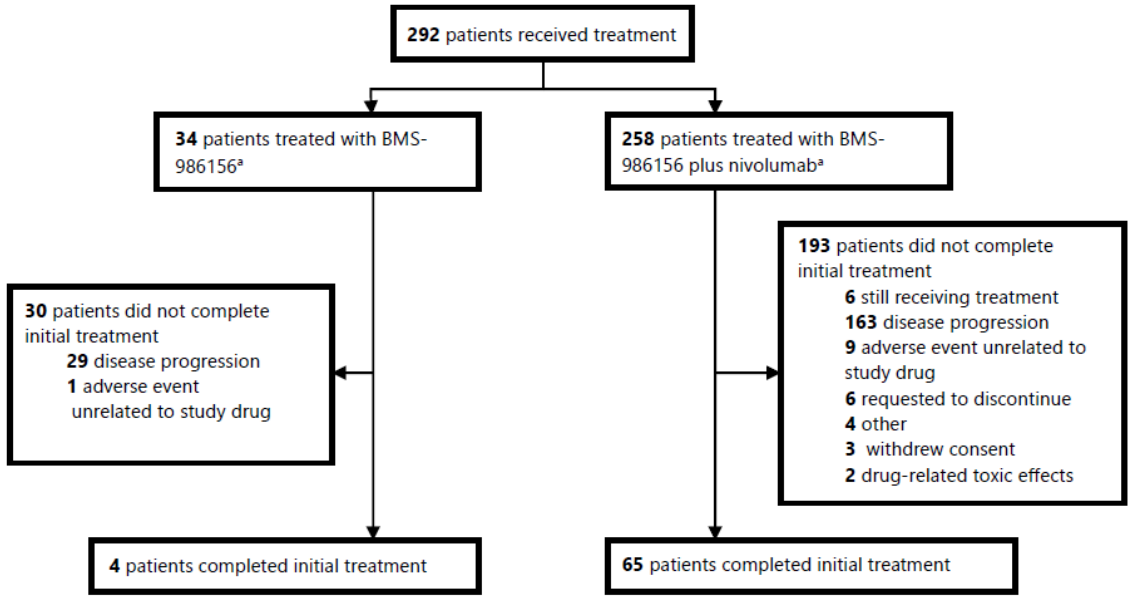
Patients

Twenty-seven sites across Australia, Belgium, Canada, France, Germany, Italy, the Netherlands, Spain, Switzerland, and the United States participated in this study, which began October 16, 2015, and is ongoing. Eligible patients were aged 18 years or older with confirmed, previously treated advanced solid tumors per Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 and must have received and then progressed on or been intolerant to 1 or more standard treatment regimens in the advanced or metastatic setting, if such a therapy existed. Other key eligibility criteria included an Eastern Cooperative Oncology Group performance status of 1 or less. Prior anti–PD-1 therapy or anti–PD-L1 therapy was allowed. The study was conducted in compliance with the trial protocol *(Supplement 1,* available at JAMA Oncology online*)*. The protocol, any amendments, and the patient informed consent form were reviewed and approved by an institutional review board or independent ethics committee (Australia: Linear Clinical Research Ltd, Liverpool Cancer Therapy Center, Princess Alexandra Hospital, Westmead Hospital; Belgium: Universitair Ziekenhuis Gent; Canada: Cross Cancer Institute, Princess Margaret Cancer Centre; France: Centre Claudius Regaud, Institut Curie, Institut Gustave Roussy; Germany: Klinikum Der Albrecht-Ludwigs-Universitat, Med. Klinik Und Poliklinik D. Uni Wuerzburg, Universitaetsklinikum Bonn; Italy: Istituto di Ricovero e Cura a Carattere Scientifico Istituto Nazionale dei Tumori Milano, Istituto Europeo Di Oncologia; Netherlands: NKI AVL; Spain: Fundacion Jimenez Diaz, Hosp Univer 12 De Octubre; Switzerland: Cantonal Hospital St Gallen, University Hospital Zurich; United States: Emory University, Providence Portland Medical Center, The Ohio State University, The West Clinic P.C., Thomas Jefferson University Hospital, University of California San Diego Moores Cancer Center, University of Alabama at Birmingham) prior to initiation of the study. Patients provided written informed consent.

Study Design and Treatment

This phase 1/2a, open-label study investigated BMS-986156 as monotherapy and in combination with nivolumab (study design, doses, and Consolidated Standards of Reporting Trials [CONSORT] diagram shown in *Figure 1*; trial protocol in *Supplement 1,* available at JAMA Oncology online; and *eFigure 1* in *Supplement 2,* available at JAMA Oncology online).

Figure 1. CONSORT diagram.



[a] This was not a randomized trial and the monotherapy and combination therapy arms were not comparator arms.
CONSORT indicates Consolidated Standards of Reporting Trials.

Study Outcomes

The primary objective of this study was to determine the safety, tolerability, dose-limiting toxic effects, and maximum tolerated dose of BMS-986156 either as monotherapy or in combination with nivolumab in patients with advanced solid tumors. Key secondary objectives included determining the antitumor activity and characterizing the pharmacokinetic profile and immunogenicity of BMS-986156 as monotherapy and in combination with nivolumab. Exploratory end points included nivolumab pharmacokinetics, overall survival, and the pharmacodynamics of BMS-986156 plus nivolumab via peripheral blood and intratumoral biomarker analysis.

Safety

Adverse events were assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.03.32 Adverse events were evaluated throughout the study while participants were receiving treatment and until 100 days after study completion.

Tumor Response

Assessment of tumor response was observed through computed tomography and/or magnetic resonance imaging at the start of the study and every 8 weeks until disease progression or treatment termination via RECIST, version 1.1.

Statistical Analysis

Detailed information on exclusion criteria, study treatment, and statistical, pharmacokinetic, and pharmacodynamic analyses are described in the *eAppendix* in *Supplement 2,* available at JAMA Oncology online.

Results

Patient Characteristics

Detailed patient demographics are presented in *eTable 1*. Baseline characteristics were similar between the monotherapy and combination therapy cohorts. Prior anti–PD-1 or anti–PD-L1 therapy was received by 11 of 34 patients (32.4%) in the monotherapy cohort and 51 of 258 patients (19.8%) in the combination cohort.

As of March 27, 2018, 34 patients received 10 to 800 mg of BMS-986156 monotherapy every 2 weeks and 258 patients received 30 to 800 mg of BMS-986156 plus 240 mg of nivolumab every 2 weeks or 480 mg of BMS-986156 plus 480 mg of nivolumab every 4 weeks. Duration of therapy ranged from 2.0 to 61.0 weeks, with follow-up time ranging from 1.4 to 101.7 weeks. Median treatment exposure (in weeks) is presented in *eTables 2A* and *2B* in *Supplement 2,* available at JAMA Oncology online. All patients but 1 (n = 33) were able to receive 90% or more of the planned cumulative treatment dose of BMS-986156 in the dose-escalation phase (4 patients completed initial treatment), and more than 80% of patients in each dose cohort of the combination phase (n = 227) were able to receive 90% or more of the planned cumulative treatment dose of both drugs (65 patients completed initial treatment).

Safety

Overall, the safety profile of BMS-986156 was tolerable. Any treatment-related adverse events (TRAEs) by dose in the BMS-986156 monotherapy cohort and in 5% or more of patients in the BMS-986156 plus nivolumab combination cohorts, as well as any grade 3 or 4 TRAEs in either cohort, are shown in *eTables 3A* and *3B* in *Supplement 2,* available at JAMA Oncology online. Grades 3 and 4 TRAEs occurred in 24 patients (9.3%) receiving BMS-986156 plus nivolumab. No grade 5 TRAEs were observed with either treatment. In addition, no TRAEs led to treatment discontinuation in the BMS-986156 monotherapy cohort. Three TRAEs led to treatment discontinuation of BMS-986156 plus nivolumab (grade 3 colitis, infusion-related reaction, and pancreatitis, all in the cohort receiving 240 mg of BMS-986156 plus 240 mg of nivolumab). Grade 1 to 2 pyrexia was seen in 6 patients (17.6%) treated with BMS-986156 monotherapy and 28 patients (10.9%) treated with combination therapy, usually within 24 hours of infusion without associated sequelae.

eTable 1. Patient demographics and baseline characteristics in the monotherapy and combination cohorts.

Characteristic	BMS-986156 (n=34)	BMS-986156 + nivolumab (n=258)
Age, years		
Median	56.5	60
Range	28-75	21-87
ECOG PS, n (%)		
0	15 (44.1)	115 (44.6)
1	19 (55.9)	141 (54.7)
2	0	2 (0.8)[a]
Sex, n (%)		
Male	18 (52.9)	118 (45.7)
Female	16 (47.1)	140 (54.3)
Race, n (%)		
White	30 (88.2)	232 (89.9)
African-American	1 (2.9)	2 (0.8)
Asian	3 (8.8)	21 (8.1)
Other	0	3 (1.2)
Prior regimens, n (%)		
0	3 (8.8)	7 (2.7)
1	14 (41.2)	106 (41.1)
2	5 (14.7)	56 (21.7)
3	8 (23.5)	43 (16.7)
>3	4 (11.8)	46 (17.8)
Prior anti-PD-1/PD-L1, n (%)		
Yes	11 (32.4)	51 (19.8)
Tumor type, n (%)		
Bladder	0	29 (11.2)
Breast	2 (5.9)	2 (0.8)
Cervical	5 (14.7)	47 (18.2)
Colon	3 (8.8)	2 (0.8)
Gastric	0	1 (0.4)
HCC[b]	0	14 (5.4)
Head and neck[c]	0	50 (19.4)
Melanoma	6 (17.6)	1 (0.4)
NPC	0	1 (0.4)
NSCLC	3 (8.8)	39 (15.1)
Ovarian	0	44 (17.1)
Pancreatic	1 (2.9)	1 (0.4)
Prostate	1 (2.9)	1 (0.4)
Renal pelvis	0	1 (0.4)
Ureter	0	2 (0.8)
Urethra	0	1 (0.4)
Other	13 (38.2)	21 (8.1)
Not reported	0	1 (0.4)

[a] 2 patients were enrolled in the study with ECOG PS of 2, which is higher than the protocol defined limits of 0 or 1; [b] Hepatocellular carcinoma (HCC): hepatitis B virus, hepatitis C virus, non-viral; [c] Includes head and neck squamous cell carcinoma; patients with nasopharyngeal carcinoma were eligible for enrollment. ECOG PS, Eastern Cooperative Oncology Group Performance Status; NPC, nasopharyngeal carcinoma; NSCLC, non-small cell lung cancer; PD, programmed death receptor.

Any grade and grades 3 and 4 serious TRAEs occurring in the overall monotherapy and combination cohorts are shown in *eTable 4*. Grade 2 pneumonitis

was the only serious TRAE seen in patients treated with BMS-986156 monotherapy. With combination therapy, most serious TRAEs were observed in the 202 patients who received 240 mg of BMS-986156 plus 240 mg of nivolumab every 2 weeks. One dose-limiting toxic effect (grade 4 elevated creatine phosphokinase level) occurred in a patient receiving 800 mg of BMS-986156 plus 240 mg of nivolumab every 2 weeks. Causes of death in the trial are shown in *eTable 5*.

Pharmacokinetics and Immunogenicity

The pharmacokinetics for BMS-986156 monotherapy and BMS-986156 plus nivolumab combination after the first dose is shown in *eFigure 2*. Overall, the pharmacokinetics was linear and exhibited a dose-related increase in exposure that was not affected in combination with nivolumab.

eTable 4. Serious TRAEs in the BMS-986156 monotherapy and BMS-986156 plus nivolumab cohorts. BCP, blood creatinine phosphokinase; TRAEs, treatment-related adverse events.

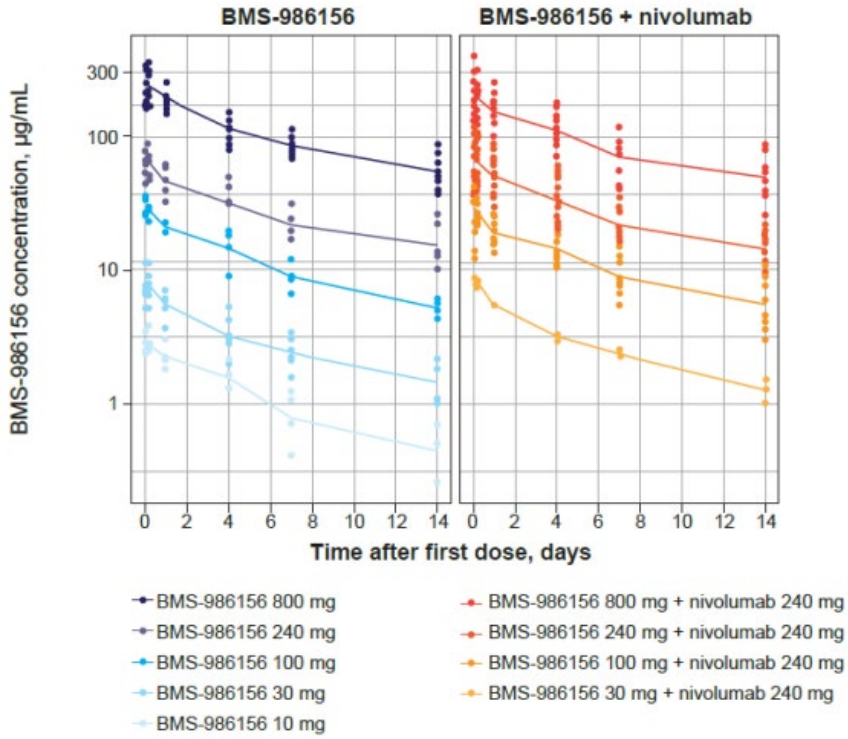
	BMS-986156 (n=34)			BMS-986156 + nivolumab (n=258)		
	Any grade, n (%)	**Grade 3-4, n (%)**	**Grade 5, n (%)**	**Any grade, n (%)**	**Grade 3-4, n (%)**	**Grade 5, n (%)**
Total patients with event, n (%)	**1 (2.9)**	**0**	**0**	**14 (5.4)**	**7 (2.7)**	**0**
Infusion-related reaction	0	0	0	4 (1.6)	1 (0.4)	0
Chronic obstructive pulmonary disease	0	0	0	1 (0.4)	1 (0.4)	0
Dehydration	0	0	0	1 (0.4)	1 (0.4)	0
Hypocalcemia	0	0	0	1 (0.4)	1 (0.4)	0
Hypokalemia	0	0	0	1 (0.4)	1 (0.4)	0
Increased BCP	0	0	0	1 (0.4)	1 (0.4)	0
Increased hepatic enzyme	0	0	0	1 (0.4)	1 (0.4)	0
Inflammation	0	0	0	1 (0.4)	0	0
Nephritis	0	0	0	1 (0.4)	0	0
Pancreatitis	0	0	0	1 (0.4)	1 (0.4)	0
Pleural effusion	0	0	0	1 (0.4)	0	0
Pyrexia	0	0	0	1 (0.4)	0	0
Type 1 diabetes	0	0	0	1 (0.4)	1 (0.4)	0
Upper abdominal pain	0	0	0	1 (0.4)	1 (0.4)	0
Pneumonitis	1 (2.9)	0	0	0	0	0

eTable 5. Causes of death.

	BMS-986156 (n=34)	BMS-986156 + nivolumab (n=258)
Death, n (%)	**27 (79.4)**	**141 (54.7)**
Disease	24 (70.6)	130 (50.4)[a]
Unknown	1 (2.9)	4 (2.8)
Airway obstruction	0	1 (0.4)
Aspiration pneumonia	0	2 (0.8)
Cardiorespiratory arrest	0	1 (0.4)
Euthanasia	1 (2.9)	1 (0.4)
Massive hemorrhage	0	1 (0.4)
Septic shock	0	1 (0.4)

[a] Discrepancy between death events owing to disease and grade 5 disease progression owing to differences in reporting – not all deaths owing to disease progression were coded as grade 5 events.

eFigure 2. Pharmacokinetics of BMS-986156 and BMS-986156 plus nivolumab.



Concentration-time profile pharmacokinetic analysis of BMS-986156 monotherapy or combination with 240 mg nivolumab after cycle 1 at all escalation phase doses. The geometric mean (CV %) values of BMS-986156 after first dose of 10 mg to 800 mg ranged from 3.03 (17.6) - 234.8 (23.9) µg/mL for Cmax, and 374.8 (29.4) - 32831.7 (21.3) µg.hr/mL for AUCTAU, when given as monotherapy.

AUCTAU, area under the concentration-time curve for 1 dose interval; Cmax, maximum serum concentration; CV, coefficient of variation.

Immunogenicity with BMS-986156 monotherapy was low; only 1 of 31 patients (at the dose of 10 mg every 2 weeks) was antidrug antibody (ADA) positive, and no patients had persistent ADA positivity (*eTable 6A*). In the combination cohort, the frequency of anti-GITR antibodies at all tested doses of BMS-986156 remained low, with 223 of 229 patients (97.4%) remaining ADA negative, and no persistently ADA-positive patients. Immunogenicity with nivolumab was relatively higher, with 35 of 223 ADA-positive patients (15.7%), and 1 of 223 patients (0.4%) persistently positive, which is consistent with nivolumab monotherapy (*eTable 6B*).

eTable 6A. Immunogenicity in the BMS-986156 monotherapy cohort by dose. ADA, anti-drug antibody; ADA positive, 4-fold higher than baseline ADA; PP, persistent positive.

	Escalation: BMS-986156						Expansion: BMS-986156		
Patients, n (%)	**10 mg (n=4)**	**30 mg (n=4)**	**100 mg (n=4)**	**240 mg (n=5)**	**800 mg (n=9)**	**All (n=26)**	**240 mg (n=3)**	**800 mg (n=2)**	**All (n=5)**
Baseline ADA positive	0	0	0	0	0	0	0	0	0
ADA positive	1 (25)	0	0	0	0	1 (3.8)	0	0	0
PP	0	0	0	0	0	0	0	0	0
Not PP, last sample positive	1 (25)	0	0	0	0	1 (3.8)	0	0	0
Other positive	0	0	0	0	0	0	0	0	0
ADA negative	3 (75)	4 (100)	4 (100)	5 (100)	9 (100)	25 (96.2)	3 (100)	2 (100)	5 (100

eTable 6B. Immunogenicity in the BMS-986156 plus nivolumab cohorts by dose. ADA, anti-drug antibody; ADA positive, 4-fold higher than baseline ADA; Nivo, nivolumab; PP, persistent positive.

	BMS-986156 30 mg + nivolumab 240 mg		BMS-986156 100 mg + nivolumab 240 mg		BMS-986156 240 mg + nivolumab 240 mg		BMS-986156 800 mg + nivolumab 240 mg		BMS-986156 480 mg + nivolumab 480 mg		All patients	
Patients, n (%)	**BMS-986156 (n=3)**	**Nivo (n=3)**	**BMS-986156 (n=8)**	**Nivo (n=8)**	**BMS-986156 (n=185)**	**Nivo (n=179)**	**BMS-986156 (n=11)**	**Nivo (n=11)**	**BMS-986156 (n=22)**	**Nivo (n=22)**	**BMS-986156 (n=229)**	**Nivo (n=223)**
Baseline ADA positive	0	0	0	1 (12.5)	1 (0.5)	16 (8.9)	0	0	0	0	1 (0.4)	17 (7.6)
ADA positive	1 (33.3)	0	0	0	5 (2.7)	34 (19)	0	1 (9.1)	0	0	6 (2.6)	35 (15.7)
PP	0	0	0	0	0	1 (0.6)	0	0	0	0	0	1 (0.4)
Not PP, last sample positive	1 (33.3)	0	0	0	3 (1.6)	11 (6.1)	0	1 (9.1)	0	0	4 (1.7)	12 (5.4)
Other positive	0	0	0	0	1 (1.1)	22 (12.3)	0	0	0	0	2 (0.9)	22 (9.9)
ADA negative	2 (66.7)	3 (100)	8 (100)	8 (100)	180 (97.3)	145 (81)	11 (100)	10 (90.9)	22 (100)	22 (100)	223 (97.4)	188 (84.3)

Pharmacodynamics

In analyzed patients, there was a trend toward an increase in $CD8^+$ T-cell and natural killer–cell proliferation after administration of anti-GITR monotherapy tested up to a dose of 240 mg every 2 weeks, although patient numbers were small (*eFigure 3*). Enhanced $CD8^+$ T-cell and natural killer–cell proliferation was observed in the combination cohort.

In the subset of patients with data available at baseline and all analyzed time points, results of flow cytometry revealed no clear depletion of regulatory T-cells in the peripheral blood in response to BMS-986156 plus nivolumab (*eFigure 4* in

Supplement 2, available at JAMA Oncology online). In addition, $CD8^+$ T-cells and $FoxP3^+$ regulatory T-cells as assessed by immunohistochemistry revealed interpatient variability from before treatment to the treatment period with BMS-986156 plus nivolumab *(Figure 2)*.

Preliminary Clinical Activity

Response results for the BMS-986156 monotherapy cohort and the BMS-986156 plus nivolumab combination cohort are summarized in *Table 1* and *Table 2*, and response results by tumor type for the combination treatment cohort are summarized in *eTable 7* in *Supplement 2,* available at JAMA Oncology online. No complete or partial responses were observed with BMS-986156 alone. Objective response rates ranged from 0% to 11.1% (1 of 9) across combination therapy cohorts; an objective response rate of 9.0% (18 of 200) was observed in the patient cohort evaluable for response who received 240 mg of BMS-986156 plus 240 mg of nivolumab.

Table 1. Efficacy in the BMS-986156 monotherapy cohort. BOR, best overall response; CR, complete response; DCR, disease control rate; NE, not evaluable; ORR; objective response rate; PD, progressive disease; PR, partial response; SD, stable disease.

Outcome	10 mg BMS-986156 (n=4)	30 mg BMS-986156 (n=6)	100 mg BMS-986156 (n=4)	240 mg BMS-986156 (n=9)	800 mg BMS-986156 (n=11)
BOR, n (%)					
CR	0	0	0	0	0
PR	0	0	0	0	0
SD	2 (50)	1 (16.7)	2 (50)	2 (22.2)	4 (36.4)
PD	1 (25)	3 (50)	2 (50)	6 (66.7)	6 (54.5)
NE	1 (25)	2 (33.3)	0	1 (11.1)	1 (9.1)
Confirmed ORR, % [95% CI]	0 [0.0-60.2]	0 [0.0-45.9]	0 [0.0-60.2]	0 [0.0-33.6]	0 [0.0-28.5]
Confirmed DCR, n (%) [95% CI]	2 (50) [6.8-93.2]	1(16.7) [0.4-64.1]	2 (50) [6.8-93.2]	2 (22.2) [2.8-60.0]	4 (36.4) [10.9-69.2]

Table 2. Efficacy in the BMS-986156 plus nivolumab combination cohort. BOR, best overall response; CR, complete response; DCR, disease control rate; NE, not evaluable; ORR; objective response rate; PD, progressive disease; PR, partial response; SD, stable disease.

Outcome	30 mg BMS-986156 + 240 mg nivolumab (n=3)	100 mg BMS-986156 + 240 mg nivolumab (n=9)	240 mg BMS-986156 + 240 mg nivolumab (n=200)	800 mg BMS-986156 + 240 mg nivolumab (n=11)	480 mg BMS-986156 + 480 mg nivolumab (n=3)
BOR, n (%)					
CR	0	0	2 (1)	0	0
PR	0	1 (11.1)[a]	16 (8)[b,c]	1 (9.1)	1 (3.4)
SD	1 (33.3)	2 (22.2)	65 (32.5)	5 (45.5)	11 (37.9)
PD	2 (66.7)	4 (44.4)	95 (47.5)	4 (36.4)	12 (41.4)
NE	0	2 (22.2)	14 (7)	0	2 (6.9)
Confirmed ORR, n (%) [95% CI]	0 [0.0-70.8]	1 (11.1) [0.3-48.2]	18 (9) [5.4-13.9]	1 (9.1) [0.2-41.3]	1 (3.4) [0.1-17.8]
Confirmed DCR, n (%) [95% CI]	1 (33.3) [0.8-90.6]	3 (33.3) [7.5-70.1]	83 (41.5) [34.6-48.7]	6 (54.5) [23.4-83.3]	12 (41.4) [23.5-61.1]

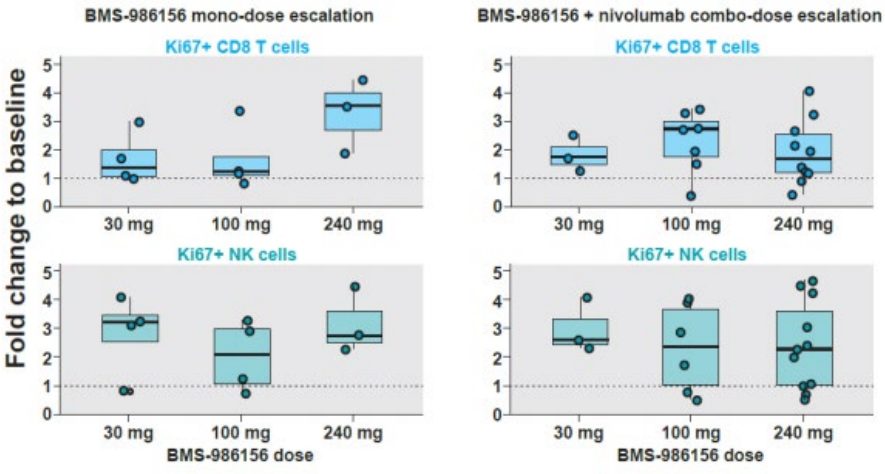
Glucocorticoid-induced TNF receptor–related protein agonist alone and in combination with nivolumab.

[a] This patient had prior treatment with pembrolizumab, with a BOR of PD, ending 111 weeks before initiation of the study treatment.

[b] One of these patients had prior treatment with nivolumab, with a BOR of PD, ending 4 weeks before initiation of the study treatment.

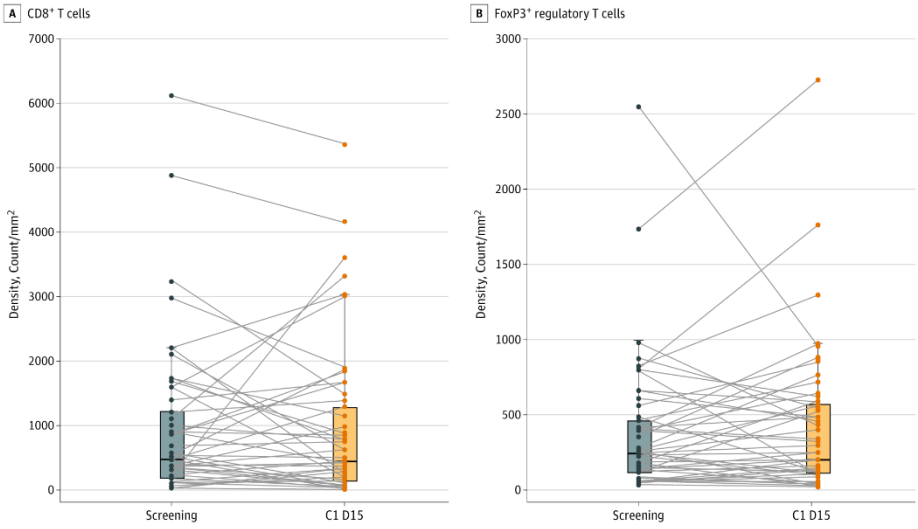
[c] One of these patients had prior treatment with pembrolizumab, with a BOR of PR, ending 83 weeks before initiation of study treatment.

eFigure 3. Peripheral CD8⁺ T-cell and Natural Killer (NK)-cell proliferation in response to BMS986156 monotherapy and BMS-986156, alone and in combination with nivolumab therapy.



The frequency of proliferating (Ki67⁺) CD8⁺ T-cells and NK-cells in the periphery was analyzed by flow cytometry for patients receiving BMS-986156 monotherapy or BMS-986156 plus nivolumab combination in the escalation part of the trial (n=11 and n=21, respectively). All patients analyzed in the combination cohort received nivolumab at a dose of 240 mg every 2 weeks. Data shown for all patients with available samples as change from baseline levels of peripheral CD8⁺ T-cell and NK-cell populations.

Figure 2. Intratumoral T-cell and regulatory T-cell modulation after BMS-986156 plus nivolumab therapy.



Tumor-infiltrating CD8⁺ T-cells (P = .72) (A) and FoxP3⁺ regulatory T-cells (P = .44) (B) by immunohistochemistry biopsies before treatment and while receiving treatment for 53 matched pairs of patients receiving BMS-986156 plus nivolumab combination therapy (all patients included in this analysis received 240 mg of nivolumab every 2 weeks). The Wilcoxon signed rank test was performed for all the comparisons. The horizontal line in each box plot denotes the median level. C1 D15 indicates cycle 1, day 15.

Discussion

Here, results are presented from a phase 1/2a dose-escalation and dose-expansion study of the GITR agonistic antibody BMS-986156 with or without nivolumab in 292 patients with advanced solid tumors. Overall, the safety profile of BMS-986156 monotherapy appeared to be manageable, with no unanticipated safety signals, no dose-limiting toxic effects, and no treatment discontinuations owing to TRAEs. The findings suggest that the safety profile of BMS-986156 plus nivolumab at all doses was manageable and tolerable and was similar to that of nivolumab alone.[2,33,34] The rate of infusion-related reactions with combination therapy was the only TRAE that potentially appeared more frequently than observed with nivolumab alone.[2,33,34]

Limitations

Some limitations of this trial include the absence of comparator groups and the enrollment of an unselected population. For example, although combination therapy with BMS-986156 plus nivolumab yielded pharmacodynamic changes and clinical response rates similar to those historically observed with nivolumab monotherapy in patients with advanced solid tumors (range, 13%-20%),[2,33-35] this trial was not designed to be a head-to-head comparison of nivolumab monotherapy vs nivolumab plus BMS-986156. Thus, without an appropriate comparator group (eg, nivolumab monotherapy), dissecting the effects of BMS-986156 and nivolumab is difficult, and the contribution of the GITR agonist to the combinatorial clinical activity remains unclear. In addition, no tumor type appeared to respond more favorably to combination therapy vs others. Most observed responses occurred in patients without prior anti–PD-1 or anti–PD-L1 therapy, although a few responses were observed in patients with prior anti–PD-1 or anti–PD-L1 therapy. Overall, however, the data appear to indicate the absence of substantial clinical activity of BMS-986156 in an unselected, broad population of patients with advanced solid tumors.

Similar observations of a tolerable safety profile and some immunomodulatory action, but limited clinical activity, have been made with other GITR agonist antibodies in clinical trials; across 4 other studies, more than 100 patients have been treated with GITR agonist monotherapy, demonstrating a manageable safety profile and limited clinical activity (the best overall response observed was stable disease).[19,21,22,36] Based on current available data, to date, no synergistic activity has been observed between GITR agonists and pembrolizumab when administered as a combination treatment, except potentially in checkpoint inhibitor–naive patients with melanoma, based on a small cohort of 13 patients.[21,37]

Although checkpoint inhibitors have made strides in revolutionizing cancer therapy, to our knowledge, there has not been a clear path identified to date for

costimulatory therapy combinations. Other members of the TNFR superfamily and classes of costimulatory molecules that are mechanistically different from TNFR are also under investigation, including inducible costimulator, a member of the CD28 family, which promotes T-cell proliferation and cytokine production after T-cell activation.[15] The potential role of combination checkpoint inhibition plus agonistic induction of costimulatory T-cell pathways in certain patient subsets with immunotherapy-resistant or refractory tumors remains to be determined.

Conclusions

To our knowledge, this represents the largest clinical data set investigating GITR agonist therapy (BMS-986156) with or without nivolumab. We believe that BMS-986156 has a manageable safety profile, and its combination with nivolumab seems to show a similar safety signal to that of nivolumab monotherapy. Clinical activity of anti-GITR therapy plus anti–PD-1 therapy was similar to historically observed activity with anti–PD-1 therapy alone. Thus, no evidence of monotherapeutic clinical activity for GITR agonism was observed in this broad population. In addition, no clear signal has emerged to date demonstrating that GITR agonism may be an effective therapeutic strategy in a broad patient population.

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CHAPTER 4.2

OX40 Agonist BMS-986178 Alone or in Combination With Nivolumab and/or Ipilimumab in Patients With Advanced Solid Tumors.

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Submitted to Clinical Cancer Research on 20 May 2020.
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Abstract

Purpose: This phase 1/2a study (NCT02737475) evaluated the safety and activity of BMS-986178, a fully human OX40 agonist immunoglobulin G1 monoclonal antibody, ± nivolumab and/or ipilimumab in patients with advanced solid tumors.

Experimental Design: Patients (with non-small cell lung, renal cell, bladder, other advanced cancers) received BMS-986178 (20–320 mg) ± nivolumab (240–480 mg) and/or ipilimumab (1–3 mg/kg). The primary endpoint was safety. Additional endpoints included immunogenicity, pharmacodynamics, pharmacokinetics, and antitumor activity per RECIST version 1.1.

Results: Twenty patients received BMS-986178 monotherapy, and 145 received combination therapy in various regimens (including 2 patients receiving nivolumab monotherapy). With a follow-up of 1.1 to 103.6 weeks, the most common (≥ 5%) treatment-related adverse events (TRAEs) included fatigue, pruritus, rash, pyrexia, diarrhea, and infusion-related reactions. Overall, grade 3–4 TRAEs occurred in 1 of 20 patients (5%) receiving BMS-986178 monotherapy, 6 of 79 (8%) receiving BMS-986178 plus nivolumab, 0 of 2 receiving nivolumab monotherapy, 6 of 41 (15%) receiving BMS-986178 plus ipilimumab, and 3 of 23 (13%) receiving BMS-986178 plus nivolumab plus ipilimumab. No deaths occurred. No dose-limiting toxicities were observed with monotherapy, and the maximum tolerated dose was not reached in either the monotherapy or the combination escalation cohorts. No objective responses were seen with BMS-986178 alone; objective response rates ranged from 0% to 13% across combination therapy cohorts.

Conclusion: In this study, BMS-986178 ± nivolumab and/or ipilimumab appeared to have a manageable safety profile, but no clear efficacy signal was observed above that expected for nivolumab and/or ipilimumab.

Statement of Translational Relevance

Immune checkpoint inhibitors have improved the treatment of several cancers, but novel approaches are needed to extend benefits to more patients and to enhance the duration of response. Combination of immune checkpoint inhibitors with OX40.23, a murine ligand-blocking OX40 agonist, demonstrated enhanced efficacy in preclinical models. In this phase 1/2a study, BMS-986178, a fully human immunoglobulin G1 agonist monoclonal antibody, with or without nivolumab and/or ipilimumab exhibited an acceptable safety profile in patients with advanced solid tumors. Objective response rates were not higher than those that would have been expected with nivolumab with or without ipilimumab. In summary, the findings of this study in a broad population of patients with advanced cancer did not demonstrate a clear improved efficacy signal for BMS-986178 with or without nivolumab and/or ipilimumab.

Introduction

Cancer immunotherapy modulates the immune system to promote an antitumor response in patients with cancer and includes various approaches (1). One established approach is the utilization of immune checkpoint inhibition with anti–programmed death protein-1 (anti–PD-1)/anti–programmed death protein ligand 1 (anti–PD-L1) and anti–cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) monoclonal antibodies. This approach has demonstrated durable antitumor responses and significant survival benefit in many tumor types, including melanoma, bladder, and renal cell carcinoma as well as non-small cell lung cancer (NSCLC) (2-4). For example, combination of the anti–PD-1 inhibitor nivolumab and CTLA-4 inhibitor ipilimumab has led to enhanced antitumor responses and survival compared with either inhibitor alone (5-8). Despite these benefits, many patients exhibit resistance to checkpoint inhibitor therapies (3). Thus, there is a need for novel immuno-oncology strategies that modulate the immunosuppressive tumor microenvironment and enhance antitumor T-cell responses (4,5).

Activation of costimulatory pathways that stimulate T-cell response and inhibit regulatory T-cell–mediated suppression of effector CD4+ and CD8+ T cells is associated with enhanced antitumor response induced by checkpoint inhibitors in preclinical models (9). The tumor necrosis factor receptor super family (TNFRSF) includes several costimulatory proteins with key roles in T-cell development and survival, immune activation, and antitumor immune responses (9-11). Preclinical data suggest that agonistic antibodies to TNFRSF costimulatory receptors could provide therapeutic benefit and further enhance the antitumor response observed when they are combined with checkpoint inhibitors (12-15). OX40 is a member of the TNFRSF and regulates multiple T-cell functions (4,16-18). The cell surface expression of OX40 is upregulated following T-cell activation; upon binding the OX40 ligand, it provides costimulatory signals, increasing the activation of CD4+ and CD8+ T effector cells in preclinical studies (4,16,17). OX40 may also inhibit regulatory T-cell–mediated suppression and block the generation of regulatory T cells, leading to enhanced T effector cell activity (17). Anti-OX40 monotherapy suppressed tumor growth in several preclinical mouse tumor models and also enhanced antitumor T-cell activity when combined with checkpoint inhibitors, supporting the potential of these combinations to provide more durable responses than checkpoint inhibitor monotherapy (4,19-21). The role of OX40 activity is being studied in various human cancers, including bladder (22), colorectal (23,24), renal cell (22,25,26), and NSCLC (27).

BMS-986178 is a fully human immunoglobulin G1 agonist monoclonal antibody that binds with high affinity to the OX40 receptor (28). Preclinical studies with the murine analogue OX40.23 as monotherapy demonstrated antitumor activity that was

further enhanced when it was combined with checkpoint inhibitors (29). In the phase 1 clinical trial, the combination of BMS-986178 and nivolumab or ipilimumab demonstrated linear pharmacokinetics with dose-related increases in exposure (30). BMS-986178 monotherapy increased proinflammatory cytokines, such as IFN-ɣ and the IFN-ɣ–induced cytokines CXCL9 and CXCL10, in patients with advanced solid tumors, with greater effects observed after combination therapy (28). In addition, BMS-986178 treatment increased the proliferation of CD4+/CD8+ effector memory cells that was enhanced when BMS-986178 was combined with nivolumab (anti–PD-1) or ipilimumab (anti–CTLA-4) (28). Although increases in exposure corresponded with pharmacodynamic effects, as previously reported, the optimal dose of OX40 agonism may not be the highest dose possible. Dose-optimization studies demonstrated that OX40 receptor occupancy between 20% and 50% both in vitro and in vivo was associated with maximal enhancement of T-cell effector function by anti-OX40 treatment, whereas a receptor occupancy > 40% led to a profound loss in OX40 receptor expression (28). Here we describe results of a phase 1/2a dose-escalation and -expansion study of BMS-986178 with or without nivolumab and/or ipilimumab in patients with advanced solid tumors (NCT02737475).

Patients and Methods

Study design and treatment

NCT02737475 (CA012004) is an open-label phase 1/2a study investigating the safety, pharmacokinetics, pharmacodynamics, and antitumor activity of BMS-986178 as monotherapy and in combination with nivolumab and/or ipilimumab in patients with advanced solid tumors across 28 sites in Canada, France, Israel, Italy, the Netherlands, Spain, and the United States. This multicohort study is composed of dose-escalation/exploration and dose-expansion phases that evaluated BMS-986178 alone or in combination with nivolumab and or ipilimumab. Each cohort proceeded in a phased approach based on study-emergent safety, pharmacokinetic, and pharmacodynamic data. During the dose-escalation phase, patients with advanced solid tumors who were refractory to or intolerant of the established therapy known to provide clinical benefit for their disease in the advanced, recurrent, or metastatic setting were treated with 20, 40, 80, 160, or 320 mg of BMS-986178 intravenously (IV) every 2 weeks (Q2W; monotherapy; part 1A), BMS-986178 (monotherapy escalation doses) plus nivolumab 240 mg IV Q2W (part 2A), or BMS-986178 (monotherapy escalation doses) plus ipilimumab 1 mg/kg IV every 3 weeks (Q3W; part 3A; *Fig. 1*).

The rationale for selection of these doses has been described previously and was based on pharmacokinetic and pharmacodynamic modeling of the relationship between receptor occupancy, pharmacodynamic modulation, and efficacy (28-31). A

mathematical model was developed to identify a dose and schedule for BMS-986178 that would achieve receptor occupancy between 20% and 50%, thus maximizing the potentiation of T-cell responses (28,31).

Combination treatments in parts 2A and 3A were initiated after completion of ≥ 3 dose cohorts in part 1A. Dose escalation decisions were based on dose-limiting toxicities (DLTs) using a Bayesian logistic regression model (for BMS-986178 monotherapy) or a Bayesian-Copula logistic regression model (for BMS-986178 in combination with nivolumab and/or ipilimumab) along with pharmacokinetic, pharmacodynamic, immunogenicity, and safety data. The observation period to detect a DLT was 28 days for both monotherapy and combination therapy dose-escalation parts. Dose-escalation recommendations were made after DLT information became available for each dosing cohort of patients. The sample size for each dose-escalation cohort depended on observed toxicity and posterior inference. Approximately 90 patients were expected to be treated during the dose-escalation phase (BMS-986178 monotherapy [part 1A], n = 30; BMS-986178 in combination with nivolumab [part 2A], n = 30; BMS-986178 in combination with ipilimumab [part 3A], n =30).

In the dose-expansion phase, patients with advanced bladder cancer received BMS-986178 80 mg plus nivolumab 240 mg Q2W (part 2C), patients with renal cell carcinoma received BMS-986178 40 mg plus nivolumab 240 mg + ipilimumab 1 mg/kg Q3W for 4 cycles followed by maintenance BMS-986178 and nivolumab 480 mg Q4W (part 6B), and patients with NSCLC received BMS-986178 40 mg plus nivolumab 240 mg Q2W and ipilimumab 1 mg/kg every 6 weeks (part 7B; *Fig. 1*). These doses were determined following initiation of parts 1A, 2A, and 3A and evaluation of safety after initial dose escalation. The dose-expansion cohorts evaluating the combination of BMS-986178 plus nivolumab and ipilimumab were preceded by safety lead-in cohorts (parts 6A and 7A).

The estimated sample size for the dose-expansion phase was guided by a Simon 2-stage design, which was based on target response rate (target objective response rate [ORR]) and the ability to identify a signal. The total sample size for each expansion cohort was calculated to provide reasonable false-positive and false-negative rates based on assumptions of true (target) and historical ORRs for each indication (sample size determinations are described in the online Supplement). Decisions regarding continuing or not continuing enrollment in a specific arm were based on a combination of model guidance, clinical judgment on the totality of data, and the discretion of the sponsor and investigators.

In additional dose-exploration cohorts, patients were treated with BMS-986178 80 mg Q2W plus nivolumab 480 mg every 4 weeks (Q4W; part 4), BMS-986178 80 mg Q3W

plus ipilimumab 3 mg/kg Q3W (part 5), or BMS-986178 20/40/80 mg every 12 weeks plus nivolumab 480 mg Q4W or nivolumab monotherapy 480 mg Q4W (part 8).

A minimum of 6 patients (up to 12) were treated in the different dose-exploration cohorts. A sample size of 6 to 12 patients per dose level and schedule provided 90% probability of observing ≥ 1 occurrence of a specific adverse event (AE) that would occur with a 32% or 17% incidence in the population, respectively. It was assumed that this number of patients would provide an accurate estimate (within 20% of the true value) of the ratio of on-treatment to baseline pharmacodynamic biomarker values.

Patients were treated with BMS-986178 alone or in combination with nivolumab and/or ipilimumab for 24 weeks (parts 1–7) or 24 months (part 8) or until meeting protocol-specified discontinuation criteria, followed by a safety follow-up of 100 days. For part 8 and any patients in part 2, 4, 6, or 7 who were approved for additional cycles up to 2 years of treatment, patients were monitored for 2 years from the first study dose to evaluate tumor response and survival. In all study sections, patients were treated until clinical deterioration, progressive disease, unacceptable toxicity, or completion of 24 weeks of treatment. Treatment beyond progression was allowed if patients were continuing to experience clinical benefit as assessed by the investigator, tolerating treatment, and meeting other protocol-specified criteria. Patients completing approximately 24 weeks of treatment with ongoing disease control (complete response, partial response [PR], or stable disease [SD]) and without any significant toxicity were eligible for retreatment.

The study protocols were approved by the institutional review board or independent ethics committee of each participating institution. The studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice as defined by the International Council for Harmonisation. All patients provided written informed consent prior to enrollment.

Patient eligibility

Eligible patients were aged ≥ 18 years, had confirmed advanced solid tumors (metastatic, recurrent, refractory, and/or unresectable) and ≥ 1 lesion with measurable disease per Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, and had progressed on or been intolerant of ≥ 1 standard treatment regimen in the advanced or metastatic setting (parts 1A, 2A, 2E, 3A, 4, 5, 6A, 7A). However, in patients with bladder cancer (parts 2C, 8) or cervical cancer (part 2D), patients must have been offered and/or received or refused ≥ 1 prior platinum-based therapy. In part 6B, no prior therapy was allowed for patients with renal cell carcinoma (RCC), with the exception of 1 prior adjuvant or neoadjuvant therapy for completely resectable disease

that did not include an agent targeting VEGF or VEGF receptors. In part 7B, no prior systemic therapy was allowed for patients with stage IV or recurrent NSCLC and if recurrence occurred ≥ 6 months after the last dose. Other key eligibility criteria included Eastern Cooperative Oncology Group performance status of 0 or 1. Patients were required to provide pre- and on-treatment tumor biopsies (core needle, excisional, or incisional). Prior immune checkpoint inhibitor therapy was permitted following a washout period > 4 weeks after the last treatment. Specific eligibility criteria for dose-expansion and -exploration cohorts are described in the online Supplement (*available at Clinical Cancer Research online)*.

Patients were excluded if they had metastases in the central nervous system or it was the only site of disease, carcinomatous meningitis, autoimmune disease, interstitial lung disease that was symptomatic and required systemic treatment with either corticosteroids (prednisone equivalents > 10 mg daily) or other immunosuppressive medications within 14 days of study drug administration, uncontrolled or significant cardiovascular disease, or history of chronic hepatitis.

Patients were also excluded if they had been treated previously with T-cell costimulation agents; had a history of life-threatening toxicity related to prior immune therapy; had evidence of active infection, positive HIV test history, or known AIDS; or had any major surgery within 4 weeks of study drug administration.

Study objectives and assessments

The primary objective of this trial was to determine the safety, tolerability, DLTs, and maximum tolerated dose (MTD)/recommended phase 2 dose of BMS-986178 administered alone or in combination with nivolumab and/or ipilimumab in patients with advanced solid tumors. AEs were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03 during treatment and for ≥ 100 days after the last dose of study treatment. Secondary objectives included antitumor activity, immunogenicity, pharmacokinetics, and pharmacodynamics. Exploratory endpoints included the change from baseline in biomarker measurements of peripheral blood and/or tumor tissue. Disease status was assessed by investigators using computed tomography scans and/or MRI at baseline and every 8 weeks (± 1 week), followed by every 12 weeks during the response follow-up phases per RECIST v1.1 (32) until discontinuation of treatment or withdrawal from the study.

Serum samples for pharmacokinetics and immunogenicity assessments were collected from all patients receiving BMS-986178 alone or in combination with nivolumab and/or ipilimumab. Pharmacokinetics was characterized by noncompartmental analysis. Immunogenicity analyses for antidrug antibodies (anti–

BMS-986178 and/or anti-nivolumab and/or anti-ipilimumab) were performed using validated immunoassays. An assay to measure total soluble OX40 in patient serum was developed and validated (fit for purpose) using the Meso Scale Discovery platform. Immunohistochemistry for biomarkers, including FoxP3 (clone 23A/E7), CD8 (clone C8/144B), PD-L1 (clone 28-8), and OX40 (clone ACT35), was performed by Mosaic Laboratories on 4-mm–thick formalin-fixed, paraffin-embedded sections of tumor biopsy tissue using a BOND RX platform (Leica Biosystems). PD-L1 expression was assessed in both tumor and immune cell peripheral blood compartments. Mandatory biopsies were obtained at screening (all parts). On-treatment biopsies were collected at cycle 2 day 1 (parts 1 and 2), cycle 1 day 15 (parts 3, 5, and 6), cycle 1 day 15 (parts 4 and 7), and cycle 1 days 15 and 78 (part 8); however, on-treatment biopsies were not mandatory during dose escalation for monotherapy.

Results

Patient baseline characteristics

From June 2016 to September 2018, a total of 165 patients with advanced tumors were treated with BMS-986178 with or without nivolumab and/or ipilimumab (*Table 1; Fig. 1*). Ninety-eight patients were treated in the dose- escalation cohorts (BMS-986178 monotherapy, n = 20; BMS-986178 plus nivolumab, n = 43; BMS-986178 plus ipilimumab, n = 35). Sixty-seven patients were treated in the dose-expansion, safety, and dose-exploration cohorts (BMS-986178 plus nivolumab, n = 18 [bladder cancer]; BMS-986178 plus nivolumab, n =12 [advanced tumors]; BMS-986178 plus ipilimumab, n = 6 [advanced tumors]; BMS-986178 plus nivolumab and ipilimumab, n = 8 [renal cell carcinoma]; BMS-986178 plus nivolumab and ipilimumab, n = 15 [NSCLC]; and BMS-986178 plus nivolumab or nivolumab monotherapy, n = 8 [bladder cancer]).

Across all cohorts, the median age ranged from 55 to 69 years. The majority of patients were male (60%) and predominantly white (93%). Most patients had received prior therapy; 38% had received ≥ 3 prior lines of therapy. Twenty-four percent of patients had received prior anti–PD-1/anti–PD-L1 and 7% had received prior anti–CTLA-4 therapy (*Table 1*). At the time of database lock (08 March 2019), the duration of follow-up for response in evaluable patients ranged from 1.14 to 103.57 weeks.

Figure 1. Study design for evaluation of BMS-986178 and nivolumab and/or ipilimumab in patients with advanced solid tumors. Dose escalation cohorts were treated with 20, 40, 80, 160, or 320 mg of BMS-986178 Q2W (monotherapy; part 1A); BMS-986178 Q2W plus NIVO 240 mg Q2W (combination; part 2A); BMS-986178 Q3W plus IPI 1 mg/kg Q3W (combination; part 3A). In the dose expansion cohorts, patients with bladder cancer were treated with BMS-986178 80 mg plus NIVO 240 mg Q2W, patients with renal cell carcinoma were treated with BMS-986178 40 mg plus NIVO 240/480 mg and IPI 1 mg/kg Q3W, and patients with NSCLC were treated with BMS-986178 40 mg plus NIVO 240 mg Q2W and IPI 1 mg/kg Q6W. In the dose exploration cohorts, patients were treated with BMS-986178 80 mg plus NIVO 480 mg Q4W or BMS-986178 80 mg Q3W plus IPI 3 mg/kg Q3W (parts 4 and 5, respectively) and BMS-986178 20/40/80 mg Q12W plus NIVO 480 mg or NIVO monotherapy 480 mg Q4W (part 8). Note: For parts 3A, 5, and 6, IPI was administered through cycle 4 only. IPI, ipilimumab; NIVO, nivolumab; Q6W, every 6 weeks; Q12W, every 12 weeks

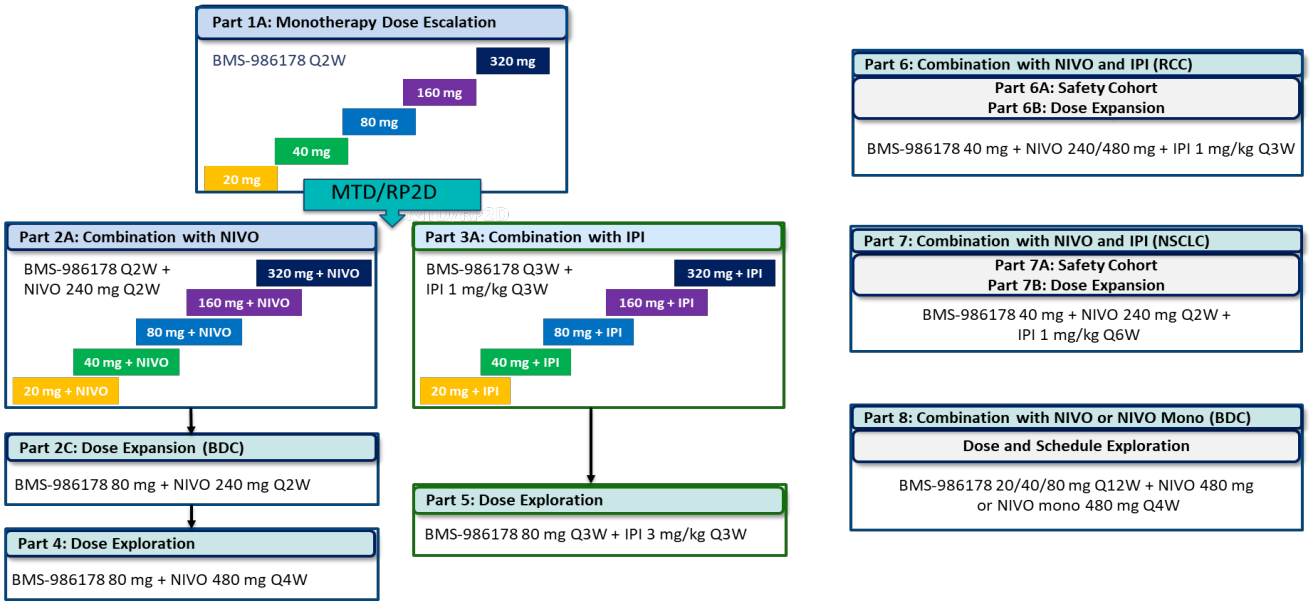


Table 1. Baseline demographics and prior therapy in patients treated with BMS-986178 and nivolumab and/or ipilimumab[a]

	Mono-therapy	Combination Therapy		Dose Expansion	Dose Exploration		Safety/Dose Expansion		Dose/Schedule Exploration
	Part 1A	Part 2A	Part 3A	Part 2C	Part 4	Part 5	Part 6A/6B	Part 7A/7B	Part 8
	BMS-986178 Q2W (n = 20)	BMS-986178 + NIVO Q2W (n = 43)	BMS-986178 + IPI Q3W (n = 35)	BMS-986178 80 mg + NIVO 240 mg Q2W (BDC) (n = 18)	BMS-986178 80 mg + NIVO 480 mg Q4W (n = 12)	BMS-986178 80 mg + IPI 3mg/kg Q3W (n = 6)	BMS-986178 40 mg + NIVO 240 mg + IPI 1 mg/kg Q3W (RCC) (n = 8)	BMS-986178 40 mg Q2W + NIVO 240/480 mg Q2W + IPI 1 mg/kg Q6W (NSCLC) (n = 15)	BMS-986178 20/40/80 mg Q12W + NIVO 480 mg Q4W and NIVO Mono (BDC)[b] (n = 8)
Median age (range), years	61 (24–80)	60 (32–82)	55 (24–79)	66 (50–80)	58 (38–70)	59 (27–73)	57.5 (25–71)	67 (56–84)	69 (60–79)
Sex, n (%)									
Male	13 (65)	23 (53.5)	14 (40)	17 (94.4)	4 (33.3)	3 (50)	6 (75)	13 (86.7)	6 (75)
Race, n (%)									
White	16 (80)	42 (97.7)	32 (91.4)	18 (100)	11 (91.7)	6 (100)	8 (100)	13 (86.7)	8 (100)
Black	2 (10)	0	0	0	1 (8.3)	0	0	1 (6.7)	0
Asian	1 (5.0)	1 (2.3)	1 (2.9)	0	0	0	0	1 (6.7)	0
American Indian or Alaska Native	0	0	1 (2.9)	0	0	0	0	0	0
Other	1 (5.0)	0	1 (2.9)	0	0	0	0	0	0
No. of prior therapies, n (%)									
0	0	2 (4.7)	1 (2.9)	0	0	0	1 (12.5)	7 (46.7)	2 (25.0)
1	8 (40.0)	7 (16.3)	16 (45.7)	11 (61.1)	5 (41.7)	1 (16.7)	4 (50.0)	8 (53.3)	4 (50.0)
2	3 (15.0)	9 (20.9)	5 (14.3)	2 (11.1)	2 (16.7)	2 (33.3)	2 (25.0)	0	1 (12.5)
≥ 3	9 (45.0)	25 (58.1)	13 (37.1)	5 (27.8)	5 (41.7)	3 (50.0)	1 (12.5)	0	1 (12.5)
Prior anti–PD-1/PD-L1, n (%)	7 (35.0)	14 (32.6)	9 (25.7)	3 (16.7)	2 (16.7)	2 (33.3)	0	1 (6.7)	1 (12.5)
Prior anti–CTLA-4, n (%)	4 (20.0)	4 (9.3)	0	2 (11.1)	0	1 (16.7)	0	0	0

a All patients had Eastern Cooperative Oncology Group performance status of 0 or 1. b Two of 8 patients in part 8 were treated with nivolumab monotherapy. Note: For parts 3A, 5, and 6, ipilimumab was administered through cycle 4 only. BDC, bladder cancer; IPI, ipilimumab; Mono, monotherapy; NIVO, nivolumab; Q6W, every 6 weeks; Q12W, every 12 weeks.

In the dose-exploration cohorts (parts 4, 5, 8), TRAEs were reported in 9 of 18 patients (50%) in the BMS 986178 plus nivolumab cohort, with 2 of 18 (11%) having grade 3 or 4 TRAEs (parts 4 and 8). In the BMS 986178 plus ipilimumab group (part 5), 3 of 6 patients (50%) had a TRAE, with 2 of 6 (33%) having grade 3–4 TRAEs. One TRAE was noted in the nivolumab monotherapy cohort (part 8). Serious TRAEs were reported in 17% of patients in parts 4 and part 8 BMS-986178 plus nivolumab (n =3/18; grade 3 duodenitis, grade 2 exacerbation of preexisting psoriatic arthropathy, grade 3 infusion-related reaction). Serious TRAEs were reported in 17% of patients in part 5 (n = 1/6; grade 3 adrenal insufficiency). TRAEs leading to discontinuation were reported in 1 patient treated with BMS986178 plus nivolumab (grade 3 duodenitis).

Overall, grade 3–4 TRAEs occurred in 1 of 20 patients (5%) receiving BMS-986178 monotherapy, 6 of 79 (8%) receiving BMS-986178 plus nivolumab, 0 of 2 receiving nivolumab monotherapy, 6 of 41 (15%) receiving BMS-986178 plus ipilimumab, and 3 of 23 (13%) receiving BMS-986178 plus nivolumab and ipilimumab (*Table 2*). No treatment-related deaths were reported.

Immunogenicity

Evaluation of the development of BMS-986178 antidrug antibodies (ADAs) during dose escalation in patients receiving monotherapy (part 1A) or BMS-986178 in combination with nivolumab (part 2A) or ipilimumab (part 3A) are shown in *Supplementary Table S1 (available at Clinical Cancer Research online)*. In patients treated with BMS-986178 monotherapy, 1 of 12 evaluated patients (8%) had developed BMS-986178 ADAs and 92% did not. In patients receiving combination therapy, 8 of 30 (27%) treated with BMS-986178 plus nivolumab developed ADAs to BMS-986178 and 11 of 26 (42%) treated with BMS-986178 plus ipilimumab developed ADAs to BMS-986178. Two of 34 patients (6%) developed ADAs to nivolumab in part 2A and 2 of 29 (7%) developed ADAs to ipilimumab in part 3A. No apparent association was observed between BMS-986178 ADAs and doses over the range of 20 to 320 mg in patients treated with BMS-986178 monotherapy.

Dose-related drug exposure

BMS-986178 exposure measured as area under the concentration vs time curve during cycle 1 was largely linear within the evaluated dose range of 20 to 320 mg for both BMS-986178 alone and in combination with nivolumab or ipilimumab (*Supplementary Fig. S1A, available at Clinical Cancer Research online*). Normalized area under the concentration vs time curve for free soluble OX40 increased with increasing BMS-986178 doses and plateaued at 160 mg (*Supplementary Fig. S1B, available at Clinical*

Cancer Research online). This observed time- and dose-dependent modulation of soluble OX40 confirmed target engagement.

Antitumor activity

No antitumor responses were observed with BMS-986178 monotherapy. The ORR was 12% (5 PRs) with BMS-986178 plus nivolumab Q2W (part 2A; cervical cancer [n = 1], RCC [n = 1], endometrial cancer [n = 1], breast cancer [n = 2]). The ORR was 6% (1 complete response) with BMS-986178 plus nivolumab Q2W (part 2C; bladder cancer) (Table 3). In the dose expansion cohorts with BMS-986178 plus nivolumab and ipilimumab, the ORR was 13% (1 PR) and 13% (2 PRs) in patients with RCC (part 6) and NSCLC (part 7), respectively. Overall, 7 of 20 patients in the BMS-986178 monotherapy cohort and 50 in the combination therapy cohorts (including nivolumab monotherapy) had SD as their best response (*Table 3*). Among 39 patients who had received prior immuno-oncology therapy, 1 had a PR and 13 had SD.

Biomarkers

The pharmacodynamic effect of treatment on proliferating ($Ki67^+$) $CD8^+$ cells and regulatory $FoxP3^+$ T- cells was interrogated by immunohistochemical analysis of paired tumor biopsy samples. Due to the protocol design, limited on-treatment tumor samples were collected in the BMS-986178 monotherapy cohort. A trend toward increased frequency of $Ki67^+CD8^+$ cells and decreased percentage of $FoxP3^+$ T-cells was observed in tumor tissue following treatment with BMS-986178 with nivolumab; however, this trend was not apparent in patients treated with BMS-986178 combined with ipilimumab or nivolumab and ipilimumab (*Fig. 2*). No consistent changes in tumor PD-L1 expression were observed during treatment with BMS-986178 and nivolumab and/or ipilimumab (*Supplementary Fig. S2, available at Clinical Cancer Research online*). At screening, 82% of tumor samples (97/118) tested had low levels (< 1%) of OX40 expression (*Fig. 3*).

Table 2. Treatment-related adverse events in patients treated with BMS-986178 and nivolumab and/or ipilimumab.

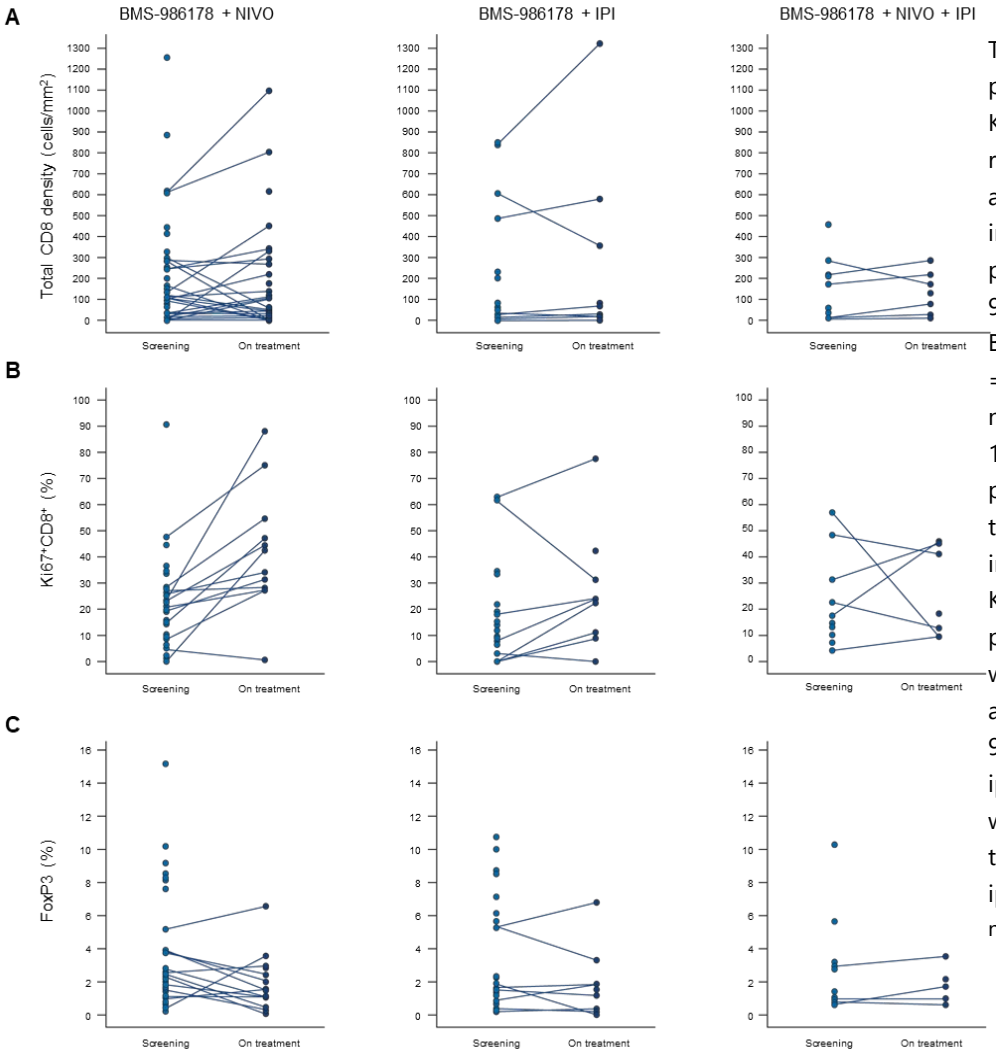
	Mono-therapy	Combination Therapy		Dose Expansion	Dose Exploration		Safety/Dose Expansion		Dose/Schedule Exploration	
	Part 1A	Part 2A	Part 3A	Part 2C	Part 4	Part 5	Part 6A/6B	Part 7A/7B	Part 8	
	BMS-986178 Q2W (n = 20)	BMS-986178 + NIVO Q2W (n = 43)	BMS-986178 + IPI Q3W (n = 35)	BMS-986178 80 mg + NIVO 240 mg Q2W (BDC) (n = 18)	BMS-986178 80 mg + NIVO 480 mg Q4W (n = 12)	BMS-986178 80 mg + IPI 3 mg/kg Q3W (n = 6)	BMS-986178 40 mg + NIVO 240 mg + IPI 1 mg/kg Q3W (RCC) (n = 8)	BMS-986178 40 mg Q2W + NIVO 240/480 mg Q2W + IPI 1 mg/kg Q6W (NSCLC) (n = 15)	BMS-986178 20/40/80 mg Q12W + NIVO 480 mg Q4W (BDC) (n = 6)	NIVO 480 mg Q4W Mono (BDC) (n = 2)
Any TRAEs, n (%)	5 (25)	21 (48.8)	18 (51.4)	12 (66.7)	6 (50.0)	3 (50.0)	5 (62.5)	12 (80.0)	3 (50)	1 (50)
Grade 1 or 2 TRAEs in ≥ 5% of patients in any cohort, n (%)[a]										
Diarrhea	1 (5.0)	2 (4.7)	1 (2.9)	1 (5.6)	1 (8.3)	0	3 (37.5)	2 (13.3)	0	0
Fatigue	1 (5.0)	5 (11.6)	3 (8.6)	5 (27.8)	2 (16.7)	3 (50.0)	3 (37.5)	3 (20.0)	0	0
Infusion-related reaction	0	1 (2.3)	5 (14.3)	0	1 (8.3)	0	0	3 (20.0)	0	0
Pruritus	0	1 (2.3)	3 (8.6)	2 (11.1)	2 (16.7)	1 (16.7)	1 (12.5)	1 (6.7)	2 (33.3)	0
Pyrexia	1 (5.0)	6 (13.9)	1 (2.9)	2 (11.1)	0	0	1 (12.5)	0	1 (16.7)	0
Rash	0	1 (2.3)	4 (11.4)	0	1 (8.3)	1 (16.7)	1 (12.5)	4 (26.7)	1 (16.7)	0
Any grade 3 or 4 TRAEs, n (%)[a]										
Adrenal insufficiency	0	0	0	0	0	1 (16.7)	0	0	0	0
Alkaline phosphatase increased	0	1 (2.3)	0	0	0	0	0	0	0	0
Asthenia	0	0	1 (2.9)	0	0	0	0	1 (6.7)	0	0
Decreased appetite	0	0	1 (2.9)	0	0	0	0	0	0	0
Diarrhea	0	0	1 (2.9)	0	0	0	0	0	0	0
Duodenitis	0	0	0	0	1 (8.3)	0	0	0	0	0
Fatigue	1 (5.0)	0	0	0	0	0	0	0	0	0

Infusion-related reaction	0	0	1 (2.9)	0	0	0	0	0	1 (16.7)	0
Lipase increased	0	0	0	1 (5.6)	0	0	0	1 (6.7)	0	0
Lymphocyte count decreased	0	0	0	1 (5.6)	0	0	0	0	0	0
Pneumonitis	0	1 (2.3)	0	0	0	0	0	0	0	0
Pruritus	0	0	0	0	0	1 (16.7)	0	1 (6.7)	0	0
Pyrexia	0	0	0	0	0	0	0	0	0	0
Rash	0	0	1 (2.9)	0	0	0	0	0	0	0
Serious TRAEs, n (%)										
Adrenal insufficiency	0	0	0	0	0	1 (16.7)	0	0	0	0
Diarrhea	0	0	1 (2.9)	0	0	0	1 (12.5)	0	0	0
Duodenitis	0	0	0	0	1 (8.3)	0	0	0	0	0
Facial paralysis	0	0	0	0	0	0	0	1 (6.7)	0	0
Infusion-related reaction	0	0	1 (2.9)	0	0	0	0	0	1 (16.7)	0
Pneumonitis	1 (5.0)	1 (2.3)	0	0	0	0	0	0	0	0
Psoriatic arthropathy exacerbated	0	0	0	0	1 (8.3)	0	0	0	0	0

a A patient could have > 1 TRAE. Note: For parts 3A, 5, and 6, ipilimumab was administered through cycle 4 only.

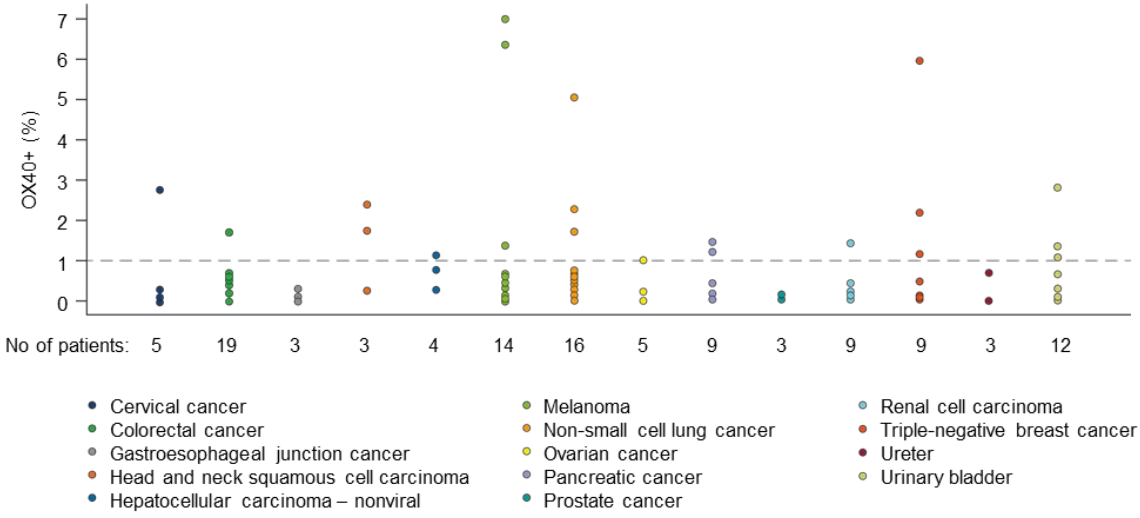
BDC, bladder cancer; IPI, ipilimumab; Mono, monotherapy; NIVO, nivolumab; Q6W, every 6 weeks.

Figure 2. Pharmacodynamics of BMS-986178 in combination with nivolumab and/or ipilimumab.



Total CD8⁺ T cells (A), percentage of proliferating Ki67⁺CD8⁺ T-cells (B), and FoxP3 regulatory T-cells (C) were analyzed by immunohistochemistry in patients treated with BMS-986178 plus nivolumab (n = 62), BMS-986178 plus ipilimumab (n = 37), and BMS-986178 plus nivolumab and ipilimumab (n = 15). Plots show individual patient values at screening vs on treatment. A trend toward increased frequency of Ki67⁺CD8⁺ cells and decreased percentage of FoxP3⁺ T-cells was observed in tumor tissue after treatment with BMS-986178 with nivolumab and/or ipilimumab; however, this trend was not apparent in patients treated with BMS-986178 plus ipilimumab. IPI, ipilimumab; NIVO, nivolumab.

Figure 3. OX40 baseline expression in various tumor types. Plot shows percentage of OX40 expression assessed by immunohistochemistry in biopsy samples from individual patients with various tumor types. Tumor type is depicted by color. Number of tumors sampled is shown below the X-axis; some symbols may overlap.



Discussion

This phase 1/2a dose escalation and expansion study evaluated the safety and preliminary antitumor activity of the OX40 agonist antibody BMS-986178 with or without nivolumab and/or ipilimumab in patients with advanced solid tumors. Overall, BMS-986178 monotherapy exhibited a tolerable safety profile, with no DLTs observed and no discontinuations due to toxicity of the study treatment. The safety of BMS-986178 demonstrated in this trial was similar to previous reports on safety from other anti-OX40 therapy clinical trials, which reported lymphopenia, fatigue, rash, infusion-related reactions, pyrexia, and pneumonitis (13,25,33-35). In the escalation cohorts, any-grade TRAEs were reported in 25% of patients receiving monotherapy and approximately 50% of patients receiving combination therapy. However, no new safety signals were observed with checkpoint inhibitors compared with monotherapy and no MTD was reached with either combination therapy regimen. Additionally, no apparent association between BMS-986178 dose and percentage of ADAs with monotherapy was observed in this patient population.

In an earlier report from this study, although there was some evidence that BMS-986178 monotherapy induced cytokines in peripheral blood (28), BMS-986178 did not appear to increase consistent changes in tumor total $CD8^+$ T-cells, proliferating $CD8^+$ T-cells, or regulatory T-cell density or provide any substantial clinical benefit in a broad population of patients with advanced solid tumors. Multiple doses, schedules, combination partners, and tumor-specific cohorts for more homogeneous patient populations were also investigated; however, no responders to monotherapy were observed. These findings suggest that the responses observed in the combination treatment groups in this study were not greater than what may have been expected with nivolumab and/or ipilimumab.

Preliminary antitumor activity has been investigated with other OX40 agonists with or without PD-L1 inhibitors, with similar results (13,25,34,35). In a phase 1 trial, monotherapy with MEDI0562, a humanized immunoglobulin G4 OX40 monoclonal antibody, resulted in limited PRs in 2 of 50 response evaluable patients (squamous cell carcinoma of the larynx and bladder cancer) (34). In another phase 1 trial, monotherapy with PF-8600, a fully human agonist immunoglobulin G2 monoclonal antibody that targets OX40, resulted in a PR only in 1 of 25 patients with advanced malignancies (25). In a preliminary report, combination of the OX40 agonist MOXR0916 and the PD-L1 inhibitor atezolizumab in a phase 1b trial in patients with advanced solid tumors demonstrated efficacy similar to that of atezolizumab monotherapy (35).

Although preclinical data demonstrated that antitumor activity with OX40 monotherapy was further enhanced with anti–PD-1 therapy, such activity was not reflected in this clinical study. Potential explanations include unknown optimal dosing

considerations, limited translation of preclinical activity in the clinic, and lack of OX40 expression at screening/baseline (82% of tumor samples [97/118] exhibited low levels [< 1%] of OX40 expression in this study; *Fig. 3*).

Optimal dosing of agonist antibodies, including those directed at T-cell agonists, is still under investigation. Antagonists are dosed to the MTD, leading to complete and sustained occupation of receptors or ligands, which may be required for maximal activity (36). However, the optimal dosing regimen for agonists may be lower or more intermittent in nature. In our previous report, OX40 receptor occupancy > 40% led to a profound loss in OX40 receptor expression in mice and humans treated every 2 weeks as well as decreased pharmacodynamic modulation (28). The doses selected in this study were predicted to produce OX40 receptor occupancy between 20% and 50%, which was associated with maximal enhancement of T-cell effector function (28). Furthermore, the optimal sequencing of checkpoint inhibitors and T-cell agonists is unclear. It has been reported that in mouse models of breast cancer, concomitant exposure to PD-1 blockade and an OX40 agonist antibody could be detrimental and that initiation of a sequential treatment with anti-OX40 was superior (37). This remains to be formally excluded, but our concomitant treatments with nivolumab did not seem to result in loss of anti–PD-1 efficacy (28).

Despite preclinical activity being observed with OX40 agonists (and other T-cell agonists)—including monotherapy activity (10,38)—these findings have not been replicated in the clinic. Although traditional xenograft and syngeneic models have been used for cytotoxic and targeted therapies, there may be limitations to these models for immunotherapy (39-41). Reasons may include but are not limited to (1) species differences in the immune system, (2) a lack of genetic, antigenic, and environmental variability in the immune systems of animal models that does not recapitulate the reality of humans, and (3) the complexity of the tumor microenvironment; the naturally occurring development of human tumors over time, including immunosurveillance, is likely a different hurdle for immunotherapy to overcome compared with that of a controlled injection of tumor cells that causes a de novo immune response at the same time that the immunotherapy is being investigated in animal models. Thus, a great need exists for improved preclinical models to allow for a more efficient and accurate assessment of novel agents to be prioritized for evaluation in the clinic.

Strategies to increase OX40 receptor expression and activated T-cells include vaccines, toll-like receptor (TLR) agents, oncolytic viruses, and radiation (42,43) and are being tested in combination in multiple clinical trials. For example, combinations of ABBV-927 (CD40 agonist) and ABBV-368 (OX40 agonist) with or without ABBV-181 (PD-1 inhibitor; NCT03893955), GSK3174998 (OX40 agonist) with pembrolizumab

(anti–PD-1; NCT03447314), and BMS-986178 with SD-101 (TLR 9 agonist; NCT03831295) are currently being evaluated.

In summary, this study demonstrated that agonism of the OX40 costimulatory receptor with BMS-986178 plus checkpoint inhibitor blockade was safe in patients with advanced malignancies but yielded no clear efficacy signal.

Acknowledgments

This study was supported by Bristol Myers Squibb, Princeton, NJ. We thank the patients and their families and the investigators and research staff at all study sites. We also thank Elena Garralda, Michel van der Heijden, Jan Schellens, Ruslan Novosiadly, and Shaun O'Brien. Editorial assistance was provided by Bridget Sackey Aboagye, PhD, and Alex Loeb, PhD, of Chrysalis Medical Communications, Inc, Hamilton, NJ, and was funded by Bristol Myers Squibb.

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Chapter 5

Fixed-dosing of monoclonal antibodies.

CHAPTER 5.1

Follow up survey for implementation of fixed-dosing of monoclonal antibodies.

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Published in International Journal of Clinical Pharmacy 2020 Feb; 42(1):3-6.
Short Research Reports.

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Abstract

Background: Similar to the earlier anti-cancer therapies, monoclonal antibodies were introduced in body-size-based schedules, despite the fact that body size only modestly effects the distribution, elimination and efficacy of monoclonal antibodies. Fixed-dosing of nivolumab and pembrolizumab has recently been approved by the European Medicines Agency.

Objective: To investigate the implementation of fixed-dosing of nivolumab, pembrolizumab and other monoclonal antibodies in the treatment of cancer.

Method: An online questionnaire was distributed among Dutch hospitals in January 2019.

Results: The majority of the hospitals (> 60%) responded, with a good representation of the characteristics of the hospitals in the Netherlands. Most hospitals which prescribe nivolumab and/or pembrolizumab have introduced fixed-dose-based schedules. However, the dosing of the other monoclonal antibodies was still based on body size.

Conclusion: Fixed-dose-based schedules of nivolumab and pembrolizumab have been rapidly implemented in most Dutch hospitals after approval of the European Medicines Agency. Despite emerging evidence which supports fixed-dose-based schedules for almost all the other monoclonal antibodies, its implementation stays behind. To increase the acceptance of fixed-dose-based schedules of monoclonal antibodies in the guidelines, additional studies may be needed, which focus on evaluating exposure, activity and cost effectiveness with the attempt to uncover the exact savings in costs for patient care.

Impact on Practice

- Fixed-dose-based schedules of monoclonal antibodies would result in increased safety (e.g. reduced dosing errors), preparation efficacy and in reduced interpatient variability.
- Additional cost savings in health care could be made, when fixed-dose-based schedules are implemented for more monoclonal antibodies.
- Implementation rates of fixed-dosing of monoclonal antibodies can be further improved.

Introduction

Monoclonal antibodies have been introduced in the field of oncology in body-size-based (e.g. in mg/kg or mg/m2) schedules. We have questioned this dosing strategy, because body size only modestly effects the distribution, elimination and efficacy of monoclonal antibodies, and proposed to change the dosing strategy from body-size-based to fixed-dosing [1]. Fixed-dosing of monoclonal antibodies would result in decreased interpatient variability and cost reductions with similar effectiveness as body-size-based dosing schedules [1–3].

Recently, the fixed-dose-based strategy has been approved by the European Medicines Agency (EMA) for the administration of nivolumab and pembrolizumab for the treatment of several malignancies [4, 5].

We have now investigated the actual introduction of fixed-dose-based schedules of nivolumab and pembrolizumab in daily practice in the Netherlands, and whether fixed-dosing is also applied for other monoclonal antibodies used in oncology.

Aim of the study

We aimed to investigate the actual introduction of fixed-dose-based schedules of nivolumab and pembrolizumab and for other monoclonal antibodies used in oncology in the Netherlands.

Ethics approval

No ethical approval was needed for this study.

Method

A short questionnaire was distributed among Dutch hospitals to obtain more insight into the use of fixed-dosing of monoclonal antibodies in oncology. This online questionnaire was distributed among 74 pharmacies of all Dutch hospitals in the beginning of January 2019. Hospital pharmacists were asked for the use of fixed-dosing and, when this was not implemented, to explain their rationale for not using fixed-dose-based schedules. A reminder was send after one month and all responses were collected until the end of February.

Results

At time of analysis, the majority of the hospitals (47; > 60%) responded with a faithful reflection of the hospitals in the Netherlands (8.9% were academic hospitals, 60% were peripheral hospitals and 30% were top clinical hospitals) [6]. Of all responding hospitals

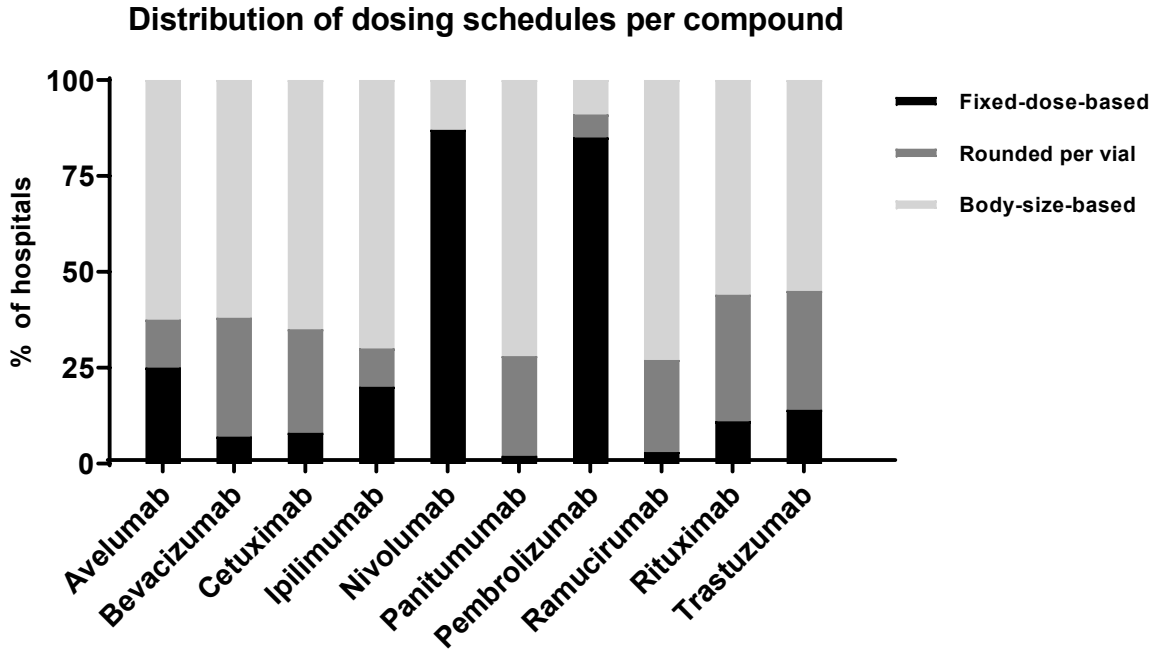
(47), 29 used nivolumab and 33 pembrolizumab. Only these hospitals were used for further analysis.

As shown in *Figure. 1* fixed-dose-based schedules of nivolumab and pembrolizumab are currently widely used. One hospital continued body-size-based schedules for nivolumab of patients being treated and new patients were treated with a fixed dose; one hospital uses body-size-based dosing until a maximum dose of 240 mg every 2 weeks (dose capping); two hospitals did not specify the rationale for maintaining body-size-based dosing for nivolumab.

In the case of pembrolizumab one hospital used body-size-based schedules because it is required in a clinical trial; two hospitals rounded per vial; one hospital uses body-size-based dosing until a maximum dose of 150 mg every 2 weeks (dose capping); one hospital did not specify the rationale for maintaining body-size-based dosing.

In contrast to nivolumab and pembrolizumab the other monoclonal antibodies are mostly dosed on the basis of body size, although some hospitals apply dose rounding to use complete vials (*Figure. 1*).

Figure 1. Fixed-dose-based versus body-size-based dosing schedule. The percentage of hospitals that administer different monoclonal antibodies in a fixed-dose-based, body-size-based schedule or rounded per vial dosing schedule. Only the hospitals that administer the monoclonal antibodies were used in the analysis.



Discussion

After the initial marketing authorization with body-size-based schedules of nivolumab and pembrolizumab in 2015 [4, 5], the transition to fixed-dosing has almost completely been implemented in the Netherlands. For other monoclonal antibodies, however, fixed-dosing is not standard treatment, despite supporting literature and a clear rationale [1, 7]. We think that this is a missed opportunity, because fixed-dosing of all monoclonal antibodies in the treatment of cancer would result in reduced interpatient variability, increased safety, increased preparation efficacy with less wastage of the product and reduced health care costs [1]. Presumably EMA approvals for the other monoclonal antibodies are awaited before moving to fixed-dosing, although there are no announcements of such steps for older monoclonal antibodies. Therefore, prescription of fixed doses might be considered as off-label use of monoclonal antibodies and the prescriber must ensure that fixed-dosing is safe and effective. This assessment should include target disposition, therapeutic window and pharmacokinetics and can be based on relevant literature, including population pharmacokinetic modeling [1].

Perhaps old habits die hard, especially when the acceptance is based on population pharmacokinetic modeling instead of a head to head comparison between a fixed-dose-based and body-size-based dosing schedule in a patient trial. In the design of new clinical trials there is more and more debate about fixed-dosing of the newly developed monoclonal antibodies. And yet, even for the quite new monoclonal antibody, ipilimumab, the administration is still adjusted to the body weight of patients. A possible explanation could be that ipilimumab has a therapeutic window that is not as wide as other monoclonal antibodies and the toxicity and efficacy are dose-dependent within the therapeutic range of ipilimumab [1]. However, based on population pharmacokinetic modeling, the dosing strategy with fixed-dosing of compounds with a significant influence of body weight on the efficacy and toxicity (like ipilimumab), is possible, by applying multiple fixed doses for cohorts with different weight ranges [1]. In fact, rounding the dose to use whole vials is a way to use multiple fixed doses. This strategy is used by several hospitals (*Figure. 1*), and, in our opinion, can be further optimized by selecting fewer weight cohorts.

An essential point in the discussion of the switch to fixed-dose-based regimens of monoclonal antibodies is the reduced interpatient variability. Furthermore, fixed-dosing could also lead to increased safety (e.g. reduced dosing errors) and increased preparation efficacy [1]. Additionally, Mukherjee [3] showed in a retrospective study that fixed-dosing was not associated with increased immune related toxicity, nor with differences in overall survival when compared with body-size-based schedules, which shows that fixed-dosing is equally effective as the weight-based dosing [3].

Another important point to address is the effect of this shift in dosing strategy on the health care costs. Three studies have attempt to predict the economic impact of fixed-dosing of pembrolizumab and nivolumab [3, 8, 9]. For pembrolizumab, Bayle [8] compared the body-size-based dose of 2 mg/kg every 3 weeks with the fixed dose of 200 mg every 3 weeks. Bayle [8] summed up the total dose of pembrolizumab which was used per dosing strategy and multiplied it with the costs per mg to look at the differences in costs. Due to the relative high dose per kg body weight with the fixed dose, the shift from a body-size-based to a fixed-dose-based regimen would result in substantial higher health care costs [8]. Mukherjee [3] retrospectively compared the costs of nivolumab 3 mg/kg every 2 weeks with the fixed dose of 240 mg every 2 weeks and of pembrolizumab 2 mg/kg every 3 weeks with the fixed dose of 200 mg every 3 weeks. Most patients were treated with a fixed dose. The patients received on average for pembrolizumab 50.6 mg more and for nivolumab 3.4 mg less per infusion with the fixed-dose-based schedules when compared with the body-size-based schedules [3]. Goldstein [9] investigated the differences in costs for pembrolizumab between fixed dose 200 mg every 3 weeks and body-size-based schedule with 2 mg/kg with a base case model. Their calculations showed that personalized dosing would result in an annual saving of 24% [9].

In comparison, other studies have stated that fixed-dosing could result in cost reductions, because there would be less wasting of the compound: complete vials could be used in the preparation; canceled prepared infusions could be used for other patients; the used dose would be relatively lower in overweight patients [1]. On the contrary, underweight patients would receive a slightly higher dose with fixed-dose-based schedules which would result in higher costs for the compound [7, 9, 10]. These factors, however, were not taken into account in the before mentioned cost analysis studies [3, 8, 9]. Additionally, the proposed fixed dose of pembrolizumab could be changed to 150 mg instead of 200 mg every 3 weeks as proposed by Hendrikx et al. [1], which was already assumed in the cost analysis in these studies [3, 8, 9]. For the introduction of fixed-dose-based regimens of monoclonal antibodies into the guidelines of the EMA, the exact dose for the fixed-dose-based schedules should of course carefully be selected and additional information about the precise impact on health care costs needs to be collected and evaluated.

Conclusion

Fixed-dose-based schedules of nivolumab and pembrolizumab have been rapidly implemented in most Dutch hospitals after approval of the EMA. Despite emerging evidence which supports fixed-dose-based schedules to almost all the other monoclonal antibodies, its implementation stays behind.

To increase the acceptance of fixed-dose-based schedules of monoclonal antibodies in the guidelines, additional studies of fixed-dosing may be needed. These studies should focus on evaluating exposure, activity and cost effectiveness with the attempt to uncover the exact savings in costs for patient care.

Acknowledgements

We would like to thank all Dutch pharmacies for their attribution to this study.

Funding

No funding was received for this study.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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CHAPTER 5.2

A cost analysis study of the implementation of fixed-dosing of monoclonal antibodies in the Netherlands Cancer Institute.

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Published online in International Journal of Clinical Pharmacy 2020, Sep 10. Research Articles.

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Abstract

Background There is a strong rationale for fixed-dosing of monoclonal antibodies in oncology. Although fixed-dosing of recently introduced monoclonal antibodies is well accepted, the rationale is also applicable for other monoclonal antibodies that already have been used for years, but are still body-size-based dosed in many hospitals. In the Netherlands Cancer Institute, Antoni van Leeuwenhoek (NKI-AVL), fixed-dosing has been implemented now for all monoclonal antibodies and, therefore, this site offers an ideal opportunity for a cost analysis study.

Objective To investigate the financial impact of switching to fixed-dosing in the NKI-AVL.

Setting The NKI-AVL.

Method Information on the preparations of monoclonal antibodies was collected from August 2017- February 2020. We compared the number of vials needed during preparation for fixed-dosing and body-size based dosing strategies. The economic impact was calculated for 2 scenarios: scenario 1 assumed clustering of all preparations per day and scenario 2 assumed no clustering of preparations.

Main outcome measure Number of saved vials and the correlating savings in health care costs.

Results The implementation of fixed-dosing resulted in a substantial reduction in vials used for almost all monoclonal antibodies. The economic savings were calculated to be €0,8 and €3,1 million per year for scenario 1 and 2, respectively.

Conclusion Fixed-dosing resulted in substantial savings in health care costs.

Impact on practice

- The implementation of fixed-dosing would result in increased safety and reduced spillage of vials.
- The implementation of fixed-dosing of all monoclonal antibodies would result in savings in health care costs.

Introduction

In the last years, there is increasing interest in fixed-dosing of monoclonal antibodies instead of body-size-based dosing (e.g. in mg/kg or mg/m2). Based on the pharmacokinetics and pharmacodynamics of monoclonal antibodies there is a strong rationale that the influence of body size on therapeutic outcome is limited [1]. Once the target of monoclonal antibodies is saturated, there is often no relation between exposure and efficacy or toxicity [1]. At the point of target saturation, also pharmacokinetic parameters, such as clearance, are minimally affected by bodyweight. Since therapeutic doses are usually much higher than doses needed for target saturation, fixed-dosing strategies are an alternative for body-size-based dosing schedules [1]. This is demonstrated by population based pharmacokinetic modelling that shows that plasma exposure of monoclonal antibodies is similar between fixed-dosing and body-size-based dosing strategies [2]. Fixed-dosing strategies are now increasingly used for monoclonal antibodies in oncology. Fixed doses are approved by the authorities for newly introduced monoclonal antibodies on the market as well as for monoclonal antibodies already having a marketing authorization for body-size-based dosing (e.g. nivolumab and pembrolizumab) [3,4]. Earlier, we showed that this fixed-dosing strategy for nivolumab and pembrolizumab was rapidly implemented in the Netherlands after registration [5]. In addition, most monoclonal antibodies in the field of oncology, which are currently under development or recently approved by the European Medicines Agency (EMA) and Food and Drug Administration (FDA), are administered in fixed doses (*Table 1*).

Table 1. overview of monoclonal antibodies that are recently registered or in late-stage development [6-10]**.**

Generic name (target)	Registered dose or late phase clinical trial	Date of registration
Atezolizumab	840 mg or 1200 mg	February 2018
Avelumab	800 mg	October 2019
Cemiplimab	350 mg	June 2019
Durvalumab	10 mg/kg	September 2018
Oportuzumab monatox	30 mg	Planned submission for registration in 2020
Margetuximab	15 mg/kg	Planned submission for registration in 2020
Relatlimab (LAG-3)	160 mg Phase II/III	Ongoing, NCT0347092
Tremelimumab	75 mg	Submitted for registration in March 2020

Although the focus of fixed-dosing is currently on the newer monoclonal antibodies, the same rationale for fixed-dosing is also valid for older monoclonal

antibodies used in oncology, which are still body-size-based dosed (e.g. bevacizumab, cetuximab, panitumumab, ramucirumab and trastuzumab). The use of fixed-dosing has advantages in terms of safety, reduction of spillage and potential cost-savings [1]. Previously, we therefore reviewed the available data and recommended fixed doses for all monoclonal antibodies used in oncology, including these older ones [1]. Based on these results, we implemented fixed-dosing strategies for all monoclonal antibodies used for solid malignancies in the Netherlands Cancer Institute, Antoni van Leeuwenhoek hospital (NKI-AVL). Fixed-dosing strategies for ipilimumab, pembrolizumab and nivolumab are used since the introduction of these drugs for routine care in our hospital. For pembrolizumab the dosing strategy changed over time, as we maximized the dose for patients over 120 kg to 200 mg every three weeks (Q3W) and introduced and optimized the every siz weeks (Q6W) schedules based on approval of 200mg fixed dose Q3W and 400mg Q6W the by EMA [3]. Simultaneously, we further optimized our Q3W schedules to the weight distribution of our patient population. For monoclonal antibodies already used in routine care in body-size-based dosing regimens, we implemented fixed-dosing for all new patients who started treatment. Patients already on treatment were transferred to fixed-dosing only after shared decision by the treating physician and the patient. We implemented fixed-dosing for bevacizumab and trastuzumab in August 2017 and for cetuximab, panitumumab and ramucirumab in April 2018. The fixed-dose-based schedules were based on previously advised doses [1] and optimized to the weight distribution in our patient population. For some monoclonal antibodies, multiple fixed doses were used, based on different weight cohorts (e.g. < or > 60 kg) or time between the infusions (e.g. every 2 or every 4 weeks). An overview of the used doses is shown in *Table 2* and *3*.

In the study of Hendrikx et al., we showed that our fixed-dosing strategy for ipilimumab, pembrolizumab and nivolumab resulted in substantial cost savings [1]. However, conflicting statements are made in literature about the actual savings in health care costs with the implementation of fixed-dose-based schedules for the monoclonal antibodies [11-15]. Most of the studies only focus on the impact of pembrolizumab and/or nivolumab on health care costs [11-15]. In addition these studies often use the registered fixed doses and do not use fixed dose schedules that are adjusted to the hospital population. This is especially important in the cost evaluation of pembrolizumab. Studies which investigate the registered dose of 200 mg show an increase in health care costs [12,14,15], while the studies using the adjusted dose of 150 mg show a decrease in health care costs [1,11,13]. Now that we have implemented fixed-dosing strategies for all monoclonal antibodies in the NKI-AVL, we wanted to evaluate the impact of our fixed-dosing schedules on vials

needed for preparations and the correlated economic impact. We hypothesized that the implementation of fixed-dosing of all monoclonal antibodies in the treatment of cancer patients could result in significant reduction of vials used and therefore would lead to substantial savings in health care costs.

Aim of the study

We aimed to investigate the financial impact of the implementation of fixed-dosing schedules for all monoclonal antibodies used in oncology in the Netherlands Cancer Institute, Antoni van Leeuwenhoek.

Ethics approval

No ethical approval was needed for this study.

Method

For our analysis, we used information from the pharmacy records about preparations used in the NKI-AVL for the treatment of patients with cancer. Since our hospital only treats patients with solid malignancies, we only had data for monoclonal antibodies used for this specific population. We extracted preparations over the period August 2017 until February 2020 and, for each preparation, we collected information about the monoclonal antibody used, the dosing strategy, the prescribed dose and the bodyweight and surface area of the patient. The information of the preparations of the monoclonal antibodies was analysed using R studio version 1.0.143 in combination with R (version 3.1.0) [16].

In total, data from 31,199 preparations was collected from the pharmacy records (see *Figure 1*). Preparations for routine clinical care were extracted by removing the preparations for clinical studies, early access programs and compassionate use programs. Preparations for cetuximab, panitumumab and ramucirumab over the period August 2017 until March 2018 were removed from the dataset since fixed-dosing for these drugs was implemented in April 2018. After grouping all preparations per compound, we sorted preparations per month. Since patients already on treatment at date of implementation of fixed-dosing continued their treatment based on body size, the dataset included also preparations for body-size-based dosing. These preparations were excluded from the dataset after checking that body-size-based dosing decreased over time and eventually all patients received fixed doses. In the end, the dataset contained 21,080 preparations for fixed-dosing strategies.

Per compound, we started the following analysis. First, we calculated for each prepared fixed dose the corresponding dose, based on body-size-based dosing (e.g. for an 80 kg patient receiving 450 mg fixed dose trastuzumab, the corresponding

body-size-based dose is 80 kg x 6 mg/kg = 480 mg). Second, we calculated the vials saved for two scenarios: scenario 1 "clustering per day", in which all preparations were clustered per day and vials needed to prepare the total daily dose at once were calculated, and scenario 2 "no clustering", in which none of the preparations were clustered per day and vials needed per preparation were calculated. For scenario 1 (clustering per day), the preparations were grouped per day, in which vials could be shared between preparations, and we calculated the number of vials needed for the preparation of both the total daily fixed dose and the total daily body-size-based dose. Last, we calculated the difference in vials needed per day for both dosing strategies and aggregated the daily differences to a total saving of vials per monoclonal antibody per year. For scenario 2 (no clustering), we calculated the vials needed for preparation of each fixed dose and for the corresponding body-size-based dose. The difference in vials needed for both dosing strategies was calculated per preparation and the calculated differences were aggregated to a total saving of vials per monoclonal antibody per year. Only the smallest available vials per compound (except for nivolumab) were used in our calculations because the price per milligram was comparable between the different vial sizes. For both scenarios (clustering per day and no clustering), the corresponding costs were based on list prices per vial in the Netherlands on February 2020 [17].

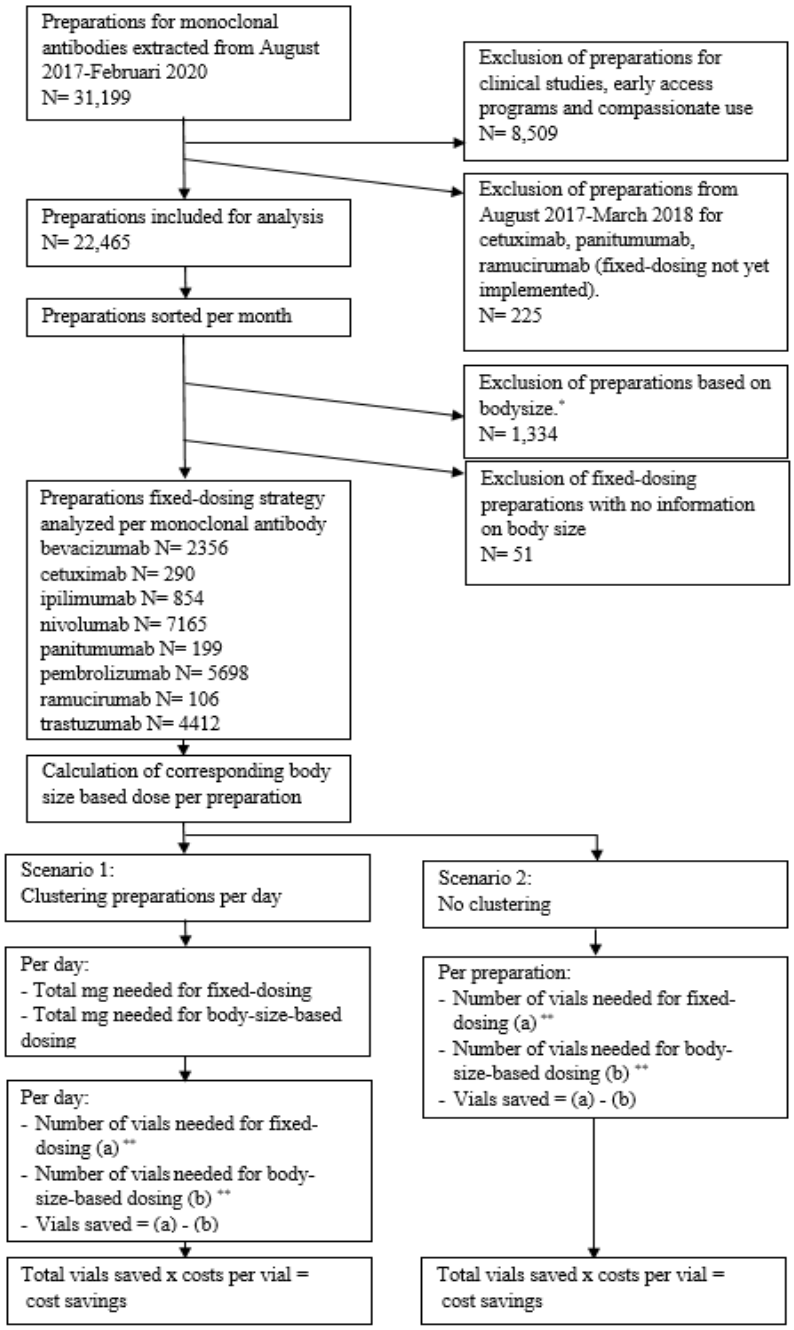
Additionally, we investigated the difference in financial impact of the registered fixed-dosing of nivolumab and pembrolizumab and the adjusted fixed-dosing scheme, which is used in the NKI-AVL.

Results

We extracted a total of 31,199 preparations of which 21,080 were eligible for our analysis (*Figure 1*). These preparations were clustered per compound. For each monoclonal antibody, we calculated the vials saved in our hospital using fixed-dosing from August 2017 until February 2020. We used two scenarios: scenario 1 involved clustering all preparations per day and scenario 2 involved no clustering. For each scenario, the number of vials needed for fixed-dosing and body-size-based dosing strategies were determined and are presented in *Table 2*.

For scenario 1 (clustering per day), the implementation of a fixed-dosing strategy resulted in a reduction of vials used for most of the monoclonal antibodies (*Table 2, Figure 2*). The fixed dose strategy, however, resulted in an increase in vials used for preparation of bevacizumab and pembrolizumab infusions when all preparations were clustered per day. Overall, the fixed dose strategy for all monoclonal antibodies resulted in savings of almost €0,8 million for scenario 1 (clustering per day), corresponding with an average saving of €33 per preparation.

Figure 1. Flowchart of data analysis.



*At date of implementation, patients already on treatment continued their treatment based on body size. This resulted in a decreasing number of preparations based on body size per month. Eventually, all preparations were based on fixed-dosing strategy.

**Number of vials is needed is based on the smallest vial available in the Netherlands Abbreviations: N = number of preparations*At date of implementation, patients already on treatment continued their treatment based on body size. This resulted in a decreasing number of preparations based on body size per month. Eventually, all preparations were based on fixed-dosing strategy.

**Number of vials is needed is based on the smallest vial available in the Netherlands.

Abbreviations: N = number of preparations.

Figure 2. Savings in costs per monoclonal antibody per year. The savings in costs with the transition of body-size-based dosing to fixed-dosing is shown. The data is separated in scenario 1: Clustering per day and scenario 2: No clustering.

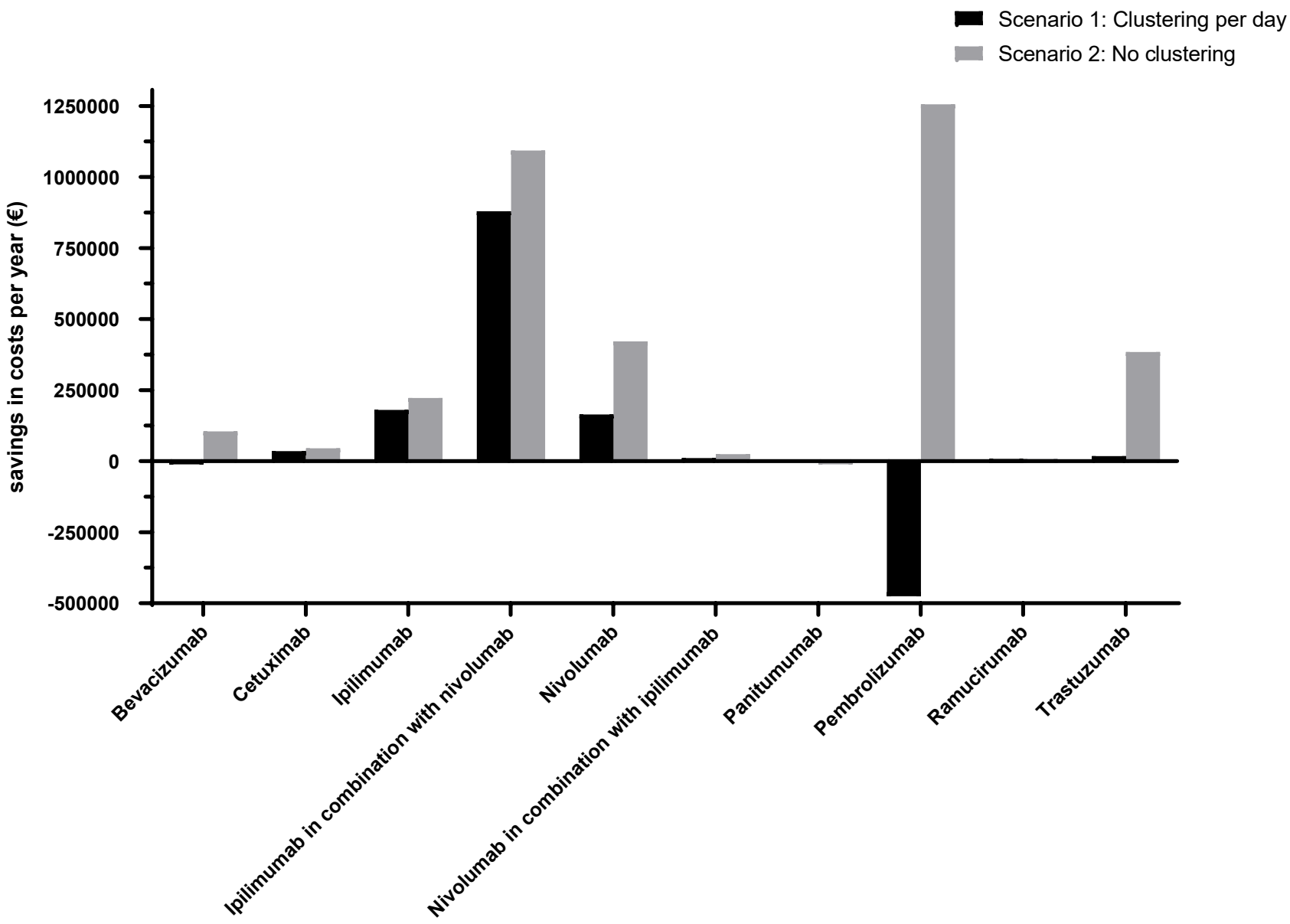


Table 2. Overview of saved number of vials and the costs with fixed-dosing. The number of vials and the correlated costs which were saved with the implementation of fixed-dosing instead of body-size-based dosing are shown. Scenario 1 clustering, in which all preparations are clustered per day, and scenario 2, in which no clustering was applied, has been calculated. Al costs were round off to whole euros. For the calculation of the total savings of vials and costs, only the most recent dosing scheme of pembrolizumab was included. LD = loading dose, N = number, α = rounded per whole vial, β = rounded of to 100 mg, *for all other indications than melanoma and non-small cell lung cancer, ** the corresponding costs were based on list prices per vial in the Netherlands [17].

						Vials saved with fixed-dosing per year		Drug substance costs saved with fixed-dosing per year and per preparation	
Generic name	**Fixed dose schemes used**	**Body-size-based schemes used**	**Period**	**N of prepa-rations**	**Vial content (costs per vial)****	**Scenario 1 Clustering per day**	**Scenario 2 No clustering**	**Scenario 1 Clustering per day**	**Scenario 2 No clustering**
Bevacizumab	400 mg Q2W 600 mg Q3W 800 mg Q2W 1200 mg Q3W	5 mg/kg Q2W 7.5 mg/kg Q3W 10 mg/kg Q2W 15 mg/kg	August 2017-February 2020	2356	100 mg (€337)	-33	312	-€11,121/ year -€11/ preparation	€105,144/ year €107/ preparation
Cetuximab	400 mg QW LD: 700 mg	250 mg/m^2 QW LD: 400 mg/m^2	April 2018-February 2020	290	100 mg (€219)	161	206	€35,259/year €233/ preparation	€45,114/year €298/ preparation
Ipilimumab	60-100 kg: 250 mg Q3W	1 mg/kg Q3W	August	222	50 mg	39	48	€180,648/	€222,336/

	<60 or >100 kg: 1 mg/kg Q3W[α]		2017-February 2020		(€4,632)			year €2,128/ preparation	year €2,566/ preparation
Ipilimumab in combination with nivolumab	<40 or >150 kg: 1 mg/kg+3 mg/kg Q3W[α] 40-70 kg: 50 mg+200 mg Q3W 70-120 kg: 100 mg+240 mg Q3W 120-150 kg: 150 mg+240 mg Q3W	1 mg/kg ipilimumab + 3 mg/kg nivolumab Q3W	August 2017-February 2020	632	Ipilimumab: 50 mg (€4,632) Nivolumab: 40 mg (€445) 100 mg (€1,112) 240 mg (€2,649) Total nivolumab	190 11 15 -4	236 29 13 -1	€880,080/ year €3,606/ preparation €4,895/year €16,680/year -€10,596/ year €45/ preparation	€1,093,152/ year €4,463/ preparation €12,905/year €14,456/year -€2,649/year €100/ preparation
	<50 or >120 kg: 3 mg/kg+ 1 mg/kg Q3W[α] 50-67 kg: 150 mg+40 mg Q3W 67-83 kg: 200 mg+80 mg Q3W 83-100 kg: 250 mg+100	3 mg/kg ipilimumab + 1 mg/kg nivolumab Q3W							

	mg Q3W 100-120 kg: 300 mg/120 mg Q3W								
Nivolumab	60-100 kg: 240 mg Q2W <60 or >100 kg: 3 mg/kg Q2W[α] 480 mg Q4W	3 mg/kg Q2W	August 2017-February 2020	6,533	40 mg (€445) 100 mg (€1,112) 240 mg (€2,649)	196 172 -43	3,772 2,846 -1,669	€87,220/year €191,264/ year -€113,907/ year	€1,678,540/ year €3,164,752/ year -€4,421,181/ year
					total	325	4,949	€164,577/ year €65/ preparation	€422,111/ year €167/ preparation
Panitumumab	<50 or >120kg: 6 mg/kg Q2W[β] 50-80 kg: 400 mg Q2W 80-120 kg: 600 mg Q2W	6 mg/kg Q2W	April 2018-February 2020	199	100 mg (€491)	1	6	€491/year €5/ preparation	€2,946/year €30/ preparation
Pembrolizuma	<55 kg: 100 mg Q3W	2 mg/kg Q3W	August	2305	50 mg	-618	199	-€933,180/	€300,490/

b	55-85 kg: 150 mg Q3W 85-120 kg: 200 mg Q3W >120 kg: 250 mg Q3W 200mg Q3W*		2017-September 2018		(€1,510)			year -€439/ preparation	year €141/ preparation
	<65 kg: 100 mg Q3W 65-90 kg: 150 mg Q3W >90 kg: 200 mg Q3W 400mg Q6W	2 mg/kg Q3W	October 2018-May 2019	1803	50 mg (€1,510)	-801	438	-€1,209,510 / year -€447/ preparation	€661,380/ year €245/ preparation
	<65 kg: 100 mg Q3W 65-90 kg: 150 mg Q3W >90 kg: 200 mg Q3W Q6W double dose	2 mg/kg Q3W	June 2019-September 2019	757	50 mg (€1,510)	-909	9	-€1,372,590/ year -€604/ preparation	€13,590/year €6/ preparation
	<65 kg: 100 mg Q3W 65-90 kg: 150 mg Q3W >90 kg: 200 mg Q3W Q6W double dose	2 mg/kg Q3W	October 2019-February 2020	833	100 mg (€2,861)	-166	439	-€474,926/ year -€238/ preparation	€1,255,979/ year €628/ preparation
Ramucirumab	≤100 kg: 600 mg Q2W >100 kg: 900 mg Q2W	8 mg/kg Q2W	April 2018-February	106	100 mg (€501)	16	16	€8,016/year €147/	€8,016/year €147/

			2020					preparation	preparation
Trastuzumab	150 mg (300 mg LD) QW 450 mg (600 mg LD) Q3W 300 mg (450 mg LD) Q2W	2 mg/kg (4 mg/kg LD) QW 4 mg/kg (6 mg/kg LD) Q2W 6 mg/kg (8 mg/kg LD) Q3W	August 2017-February 2020	4412	150 mg (€520)	34	739	€17,680/year €10/ preparation	€384,280/ year €234/ preparation
					Total	589	6,992	€811,683/ year €33/ preparation	€3,141,679/ year €342/ preparation

Table 3 Overview of saved number of vials and costs with the fixed-dosing approach of the NKI-AVL compared to the registered fixed dose. The number of vials and the correlated costs which were saved with the implementation of the adjusted schemes for fixed-dosing of nivolumab and pembrolizumab are shown. Scenario 1 clustering, in which all preparations are clustered per day, and scenario 2, in which no clustering was applied, has been calculated. Al costs were round off to whole euros. For the calculation of the total savings of vials and costs, only the most recent dosing scheme of pembrolizumab was included. N = number.

*for all other indications than melanoma and non-small cell lung cancer, ** the corresponding costs were based on list prices per vial in the Netherlands [17].

Generic name	Fixed dose schemes used in NKI-AVL	Registered fixed dose scheme	Period	N of prepa-rations	Vial content (price per vial)**	Vials saved with fixed dose scheme of NKI-AVL per year (mean per preparation)		Drug substance costs saved with fixed dose scheme of NKI-AVL per year and per preparation	
						Scenario 1 Clustering per day	Scenario 2 No clustering	Scenario 1 Clustering per day	Scenario 2 No clustering
Nivolumab	60-100 kg: 240 mg Q2W <60 or >100 kg: 3 mg/kg Q2W [α] 480 mg Q4W	240 mg Q2W 480 mg Q4W	August 2017-February 2020	6,533	40 mg (€445) 100 mg (€1,112) 240 mg (€2,649)	-106 -50 28	-161 126 72	-€47,170/ year -€55,600/ year €74,172/year	-€71,645/ year -€140,112/ year €190,728/ year
					Total	-128	37	-€28,598/ year -€11/ preparation	-€21,029/ year -€8/ preparation

Pembrolizumab	<55 kg: 100 mg Q3W 55-85 kg: 150 mg Q3W 85-120 kg: 200 mg Q3W >120 kg: 250 mg Q3W 200mg Q3W*	200 mg Q3W 400 mg Q6W	June 2018-September 2018	773	50 mg (€1,510)	834	834	€1,259,340/ year €543/ preparation	€1,259,340/ year €543/ preparation
	<65 kg: 100 mg Q3W 65-90 kg: 150 mg Q3W >90 kg: 200 mg Q3W 400mg Q6W		October 2018-May 2019	1803	50 mg (€1,510)	1,238	1,238	€1,869,380/ year €691/ preparation	€1,869,380/ year €691/ preparation
	<65 kg: 100 mg Q3W 65-90 kg: 150 mg Q3W >90 kg: 200 mg Q3W Q6W double dose		June 2019-September 2019	757	50 mg (€1,510)	1,449	1,449	€2,187,990/ year €963/ preparation	€2,187,990/ year €7963/ preparation
	<65 kg: 100 mg Q3W 65-90 kg: 150 mg Q3W >90 kg: 200 mg Q3W Q6W double dose		October 2019-February 2020	833	100 mg (€2,861)	914	643	€2,614,954/ year €1308/ preparation	€1,839,623/ year €604/ preparation

For scenario 2 (no clustering), the implementation of a fixed dose strategy resulted in a reduction of vials used for all of the monoclonal antibodies (*Table 2, Figure 2*) and resulted in substantial savings in health care costs of almost €3,1 million per year, corresponding with an average saving of €342 per preparation.

Additionally, we compared our adjusted fixed-dosing strategy with the fixed-dosing strategy approved by the competent authorities for nivolumab and pembrolizumab (*Table 3*). The adjusted fixed-dosing strategy of the NKI-AVL resulted in an additional reduction of used vials with an economic value between €1,8 and €2,6 million per year compared to the approved fixed dose, corresponding with an average saving of €668 to €604 per preparation.

Discussion

There is a strong rationale for fixed-dosing of monoclonal antibodies in oncology. Therefore, we have implemented fixed-dosing strategies for all monoclonal antibodies in the NKI-AVL, according to the previously advised dose schedules of Hendrikx *et al.* [1]. The current study aimed to evaluate the economic impact of our fixed-dosing strategy. Therefore, we determined the number of vials used per day for our fixed-dosing strategy and compared this to the number of vials used for a body-size-based dosing strategy.

We observed that a fixed-dosing strategy for monoclonal antibodies resulted in substantial cost savings. These savings were observed in both the clustering scenario as the non-clustering scenario. For the individual monoclonal antibodies, the clustering per day scenario (scenario 1) resulted in cost savings for all monoclonal antibodies, but bevacizumab and pembrolizumab. For these antibodies, the increase in costs after clustering is the result of the lower mean bodyweight of our patients and the fixed dose chosen. For pembrolizumab, the fixed dose of 150 mg corresponds to a bodyweight of 75 kg with a 2 mg/kg dosing strategy. With the high daily number of preparations, a slightly lower mean bodyweight of patients will result in one to two vials less needed for body-size-based dosing compared to fixed-dosing. For bevacizumab, the number of preparations per day is much lower. However, the fixed dose used is based on a bodyweight of 80 kg due to vial size, resulting in increased costs due to fixed-dosing after clustering all preparations per day. For both antibodies, the increase in costs is diminished when preparations are not clustered per day (scenario 2).

Because it was not practically feasible to process the information about the precise number of used vials per day, we used two scenarios to estimate the number of saved vials. For scenario 1, we assumed that all preparations were clustered per day to minimize spillage of unused vial content. For scenario 2, we assumed that no

preparations were clustered, so after every preparation there could be wastage of leftover vial content. Even in the most efficient way of using vial content (Scenario 1, clustering per day with vial sharing between preparations) the fixed dose strategy results in a substantial reduction in vials used. In routine practice, the aim is to cluster when possible, but in practice it is not always feasible to cluster all preparations in one preparation session. Since thorough microbiological, chemical and physical stability data have to be available prior to re-use vials over multiple preparation sessions, last minute changes in therapy or final approval after pre-administration checks results in additional preparations needed during the day. Therefore, the true estimation of the number of vials used and the correlated costs lies somewhere in between these two scenarios. However, it remains challenging to determine daily ratio between clustering all preparations and no vial sharing and, as a result, to estimate the exact health care savings based on these two extreme scenarios.

We showed that fixed-dosing strategies substantially reduce costs of monoclonal antibodies. Our observations are in line with previous studies that showed substantial cost savings for nivolumab and cetuximab after fixed-dosing [11-15]. However, for pembrolizumab data are more inconsistent. Some studies show cost reductions [1,11,13], while others show increased costs after the implementation of fixed-dosing [12,14,15]. These difference can be attributed to the fixed dose selected (150 or 200 mg). In Table 3 we show that our dosing strategy, in which most patients received the fixed dose of 150 mg, resulted in a cost reduction compared to the registered 200 mg fixed dose (Table 3). Furthermore, the highest savings in costs were seen after the 50 mg vial was withdrawn from the market and we had to switch to the 100 mg vials in the NKI-AVL. This is probably caused by the substantial savings in the every-six-weeks dosing regimens, in which doses of 300 or 400 mg are mostly used. This emphasizes the importance of proper dose selection by each hospital for their patient population.

The fixed doses which we have used in our institute are based on the mean bodyweight of our population and cost savings are based on our number of preparations per monoclonal antibody. Therefore, the estimated costs savings reflect our situation and may by generalized to other hospitals but with some caution. Each institute could adjust the fixed doses used to the mean bodyweight of their patient population to further optimize the economic impact of fixed-dosing. For example, when the body-size-based dose of a monoclonal antibody is 5 mg/kg and there are vials of 50 mg, a fixed dose of 350 mg would be advised in a population with a mean weight of 70 kg (70 kg*5 mg/kg= 350 mg, 350 mg/50 mg= 7 vials). But if the mean weight is 83.6 kg (United states of America) [12], a fixed dose of 400 mg would be more appropriate (83.6 kg*5 mg/kg= 418 mg, rounded of to 400 mg so complete vials

could be used, 400 mg/50 mg= 8 vials). Although our analysis shows that fixed- dosing strategies are cost-effective, selection of a hospital or region-specific dose for each monoclonal antibody could lead to the most optimal impact on drug costs as we have shown in Table 3 for our pembrolizumab schedules. Our adjusted fixed dose resulted in additional savings of €1,8 to €2,6 million per year when compared to the registered fixed dose of pembrolizumab. Unfortunately, this was not the case for nivolumab with our current dosing strategy. This was caused by having more patients in the >100 kg cohort than in the <60 kg cohort.

Overall, our analysis shows that our fixed-dosing strategy results in substantial savings in health care costs for almost all monoclonal antibodies. Moreover, the additional advantages are not yet taken into account in the presented data. Fixed-dosing enables more efficient preparation by standardizing protocols and easier preparation processes, thereby reducing preparation time. Also the risk of calculation errors is reduced, which improves the safety. Thereby, cancelled treatments can be more easily reassigned to another patient, reducing spillage of these expensive drugs and thereby further reducing healthcare costs. This also creates options for preparing infusions in advance for optimal planning. An pilot-analysis (unpublished data), which evaluated the costs of spillage by unused infusions in the NKI-AVL of the drugs mentioned in Table 2 by comparing the spillage in 2019 (fixed-dosing) with the spillage in 2015 (body-size-based dosing), shows that there was a ten times reduction in unused infusions (1.5 infusions per month compared to 16 per month respectively) with an associated cost reduction of over €25,000 per month, whereas the number of preparations of these monoclonal antibodies per month had more than doubled. These additional advantages should be taken into account when looking at the results of this study and further amplify the financial savings of fixed-dosing for all monoclonal antibodies in the treatment of solid malignancies.

Conclusion

Fixed-dosing results in substantial costs savings and can be implemented for all monoclonal antibodies in the treatment of patients with solid tumours. Adjusting fixed dose schedules to the hospital population can further increase the economic advantages. In the NKI-AVL, the implementation of our fixed-dosing strategy of all monoclonal antibodies resulted in costs savings of €0,8 to €3,1 million per year.

This study substantiates our hypothesis that the implementation of fixed-dosing of all monoclonal antibodies, in the treatment of patients with solid tumours, would help in managing the increasing health care costs with the introduction of these new and expensive monoclonal antibodies. Therefore, fixed-dosing, adjusted to the

patient population of the hospital, should be implemented for all monoclonal antibodies used in the treatment of solid tumours.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of Interest
The authors declare that they have no conflicts of interest related to this study.

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Chapter 6

Angiosarcoma.

CHAPTER 6.1

Neoadjuvant systemic treatment of primary angiosarcoma.

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Published in Cancers 2020, 12(8), 2251.
Systematic review.

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Abstract

Angiosarcoma is an extremely rare and aggressive malignancy. Standard of care of localized tumors includes surgery +/- radiation. Despite this multimodal treatment, >50% of the angiosarcoma patients develop local or distant recurrent disease. The role of neoadjuvant systemic therapy is still controversial and we therefore performed a systematic review of the literature to define the role of neoadjuvant systemic therapy based on available evidence. We focused on the effects of neoadjuvant systemic therapy on: 1. The success of surgical resection and 2. the long-term survival. All articles published before October 2019 on Ovid Medline, Ovid Embase, Cochrane library and Scopus were evaluated. Eighteen case reports and six retrospective cohort studies were included. There were no randomized controlled trials. This literature showed a beneficial role of neoadjuvant chemotherapy on downsizing of the tumor resulting in an improvement of the resection margins, especially in patients with cardiac or cutaneous angiosarcoma. However, no definitive conclusions on survival can be drawn based on the available literature lacking any prospective randomized studies in this setting. We advise that neoadjuvant chemotherapy should be considered, since this could lead to less mutilating resections and a higher rate of free resection margins. An international angiosarcoma registry could help to develop guidelines for this rare disease.

Introduction

Angiosarcoma is an aggressive sarcoma subtype, mostly deriving from endothelial cells of vascular or lymphatic origin. This neoplasm most frequently arises in (sub)cutaneous blood vessels, but can arise throughout the whole body [1]. Angiosarcoma is extremely rare and accounts for less than 1% of all soft tissue sarcomas in adults with an incidence of 1.5 per 1,000,000 persons per year [2,3]. Some case reports suggest that several familial syndromes could possibly predispose for angiosarcoma, such as a mutation in the BRCA1 or BRCA2 gene [4,5].

Angiosarcomas can be divided into different subgroups, primary (sporadic) or secondary, based on the etiology of the disease [6,7]. Primary or sporadic angiosarcoma arise from progenitor or mesenchymal stem cells anywhere in the body, but seem to have a slight predilection for the breast [7,8], while secondary angiosarcomas are mostly seen on the skin because they are caused by external damage by radiation, UV-exposure or chronic lymphedema [7]. The most common variant is the UV-induced angiosarcoma, usually arising in the skin of the face and scalp (35-62%) of mainly elderly patients [1,2,9]. Radiation associated angiosarcoma can occur anywhere in the body after previous radiation, but is most frequently seen in the breast after previous radiotherapy for a primary breast malignancy. It is estimated that around 1 in 10,000 patients per year previously treated for a malignancy with radiation, sooner or later develops angiosarcoma in the inflicted area [1,10]. Angiosarcoma in the extremity can be caused by chronic lymphedema and this disease is also known as Stewart-Treves syndrome [1,10]. The incidence of Stewart-Treves syndrome is between 1/10 and 1/20 of patients with cutaneous angiosarcoma [1]. Finally, several exogenous toxins are associated with the development of angiosarcoma, especially within the liver [11,12].The separation in primary and secondary angiosarcoma is important, because there is a difference in prognosis. Patients with secondary angiosarcoma show a better median overall survival than patients with primary angiosarcoma, 20.6 vs 7.2 months, respectively [7].

The standard of care for resectable localized disease is complete surgical resection. Despite this treatment, more than 50% of patients develop local (26-54%) or distant (>50%) recurrent disease [13,14] and only 60% of patients who initially present with localized disease survive for more than 5 years [15], meaning there is an urgent need to improve the treatment. Given this high-risk and poor prognosis of angiosarcoma, ESMO guidelines state that neoadjuvant radiation and chemotherapy may be considered [16]. Current practice regarding (neo)adjuvant treatment, however, varies widely per country and per institution. Then again, conclusive data regarding the response rates and potential survival benefit of (neo)adjuvant chemotherapy is lacking, and in modern times neoadjuvant chemotherapy is often preferred over

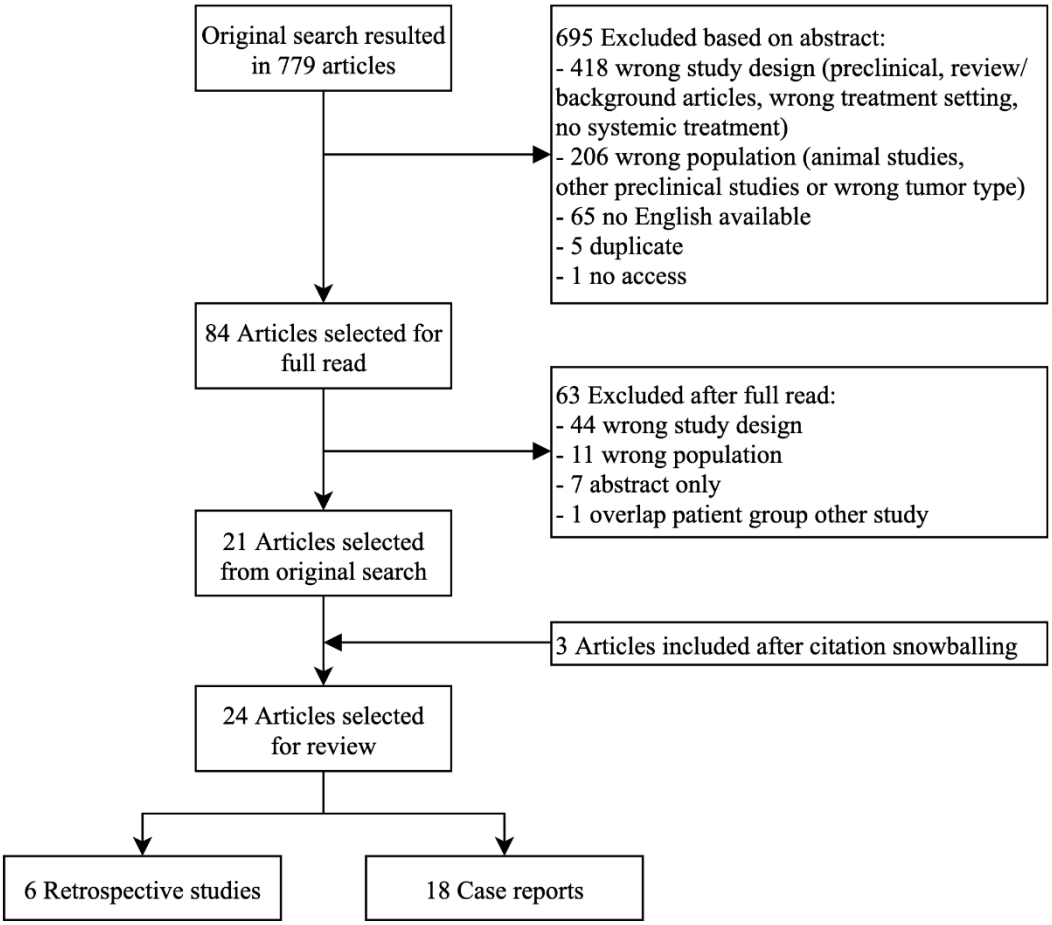
adjuvant chemotherapy to enable response evaluation and change chemotherapy regimen when no response is observed.

In general, goals of neoadjuvant systemic treatment are: 1. to facilitate adequate surgical resection by downsizing the tumor and 2. to improve survival by treating distant micrometastases, preventing outgrowth of these metastases into macrometastases. The addition of neoadjuvant systemic therapy to angiosarcoma treatment, however, is based on relatively limited available data, and consists mostly of retrospective studies and case reports. Designing a large randomized study analyzing neoadjuvant systemic therapy for angiosarcoma would be challenging, given the rarity of the disease and the different angiosarcoma subtypes with different biological behavior. With this review, we aim to provide a summary of the current literature on neoadjuvant systemic treatment of angiosarcoma. Furthermore, we aim to analyze outcome and response rates of neoadjuvant systemic therapy and evaluate tumor resectability after neoadjuvant systemic therapy. Recommendations based on available literature are given.

Results

The literature search resulted in six retrospective cohort studies and eighteen case reports with 21 individual cases discussing neoadjuvant systemic treatment (*Figure 1*). *Table 1a* and *1b* give an overview of the short-term and long-term outcome and of the effect of neoadjuvant systemic treatment on surgical margins of angiosarcoma patients in these studies. The retrospective cohort studies will first be discussed in more detail. The six retrospective cohort studies consist of one study with angiosarcoma of the face and scalp only, two studies discussing all cutaneous angiosarcoma, two studies discussing cardiac angiosarcoma and one study discussing all kinds of angiosarcoma. Secondly, the case reports will be discussed per tumor localization, because the site of origin of the disease affects the prognosis [14,15].

Figure 1. Flowchart of literature search and article selection.



*Original search: in Ovid Medline, Ovid Embase and Scopus. Terms: angiosarcoma, hemangiosarcoma and lymphangiosarcoma in combination with terms for neoadjuvant/preoperative/targeted/immuno-therapy

Table 1a. Overview of responses to neoadjuvant systemic treatment in angiosarcoma patients – retrospective cohort studies. AC= adjuvant chemotherapy, AS= angiosarcoma, CR= complete response, DFI= disease-free interval, DSS= disease specific survival, FU= follow up, NAC=neoadjuvant chemotherapy, No= number, ns= not specified, OS= overall survival, pCR= pathologic complete response, PR= partial response, pt.(s) =patient(s), refs=references, vs.=versus RT= radiotherapy.

Refs	No. of patients	Neoadjuvant treatment	Patient characteristics	Influence on resectability	Short-term response	Long-term response
[17]	33	10 pts docetaxel + gemcitabine 5 pts paclitaxel 18 pts had diverse regimens consisting of doxorubicin + ifosfamide, cyclophosphamide + doxorubicin + dacarbazine, interferon, vincristine, doxorubicin + paclitaxel or other combinations	70 pts with non-metastatic AS of face and scalp - 33 pts had NAC (regimen per pt. was ns) - 20 pts had AC - 9 pts had both	ns	- 88% response: 11 pts had CR and 18 pts had PR - 2 pts had SD (6%) - 2 pts had PD (6%)	Chemotherapy was not associated with a significant difference in OS or DSS, local or distant recurrence compared to pts who did not received chemotherapy
[13]	12	12 pts had ≥2 cycles of NAC: - Paclitaxel (n = 6) - Gemcitabine + docetaxel (n = 4) - not specified for 2 pts	23 pts with primary cutaneous or soft tissue AS	80% R0 resections after NAC (vs. 85% surgery alone)	30% had pCR (n=3, one paclitaxel, two gemcitabine+ docetaxel) - PR not specified - 2 PD during NAC (both paclitaxel)	No statistically significant survival benefit in pts who received NAC when compared to pts who did not receive NAC
[18]	38	38 pts had NAC: site of origin AS and regimens were ns 21 pts had RT	821 localized AS	ns	No short-term FU data available	Neither RT nor chemotherapy improved the OS
[19]	10	10 pts had NAC: regimens were ns	46 pts with primary cardiac sarcomas who underwent heart transplantation - 16 pts had AS	ns	No short-term FU data available	NAC did not provide survival benefit after heart transplantation compared to pts who only received heart transplantation

[20]	24	Median of 6 cycles of doxorubicin + ifosfamide or gemcitabine + docetaxel	32 pts with right sided heart sarcoma had NAC (24 with AS)	47% R0 resections after NAC (vs. 33% surgery alone)	No significant difference in the 30-day postoperative outcomes	Median survival 20 months with NAC vs 9.5 months without NAC (p=0.417). Median survival higher after R0 resection (53.5 vs. 9.5 months positive margins, P=0.004)
[14]	17	Doxorubicin +/- ifosfamide	9 pts received NAC 7 pts after R2 resection or for inoperable disease	ns	- 3 pts had CR (18%) - 7 pts PR (41%) - 2 pts SD (12%) - 5 pts PD (29%)	No significant differences in OS or PFS between pts who received NAC compared to pts without NAC

Table 1b. Overview of responses to neoadjuvant systemic treatment in angiosarcoma patients – case reports. AS= angiosarcoma, AT= adjuvant treatment, CR= complete response, CT= computed tomography, FU= follow-up, (c)Gy=(centi)gray, HIPEC= heated (hyperthermic) intraperitoneal chemotherapy, MRI= magnetic resonance imaging, NAC=neoadjuvant chemotherapy, pCR= pathologic complete response, PD= progressive disease, PDT= photodynamic therapy, PR= partial response, pt.(s)=patient(s), RFA= radio frequent ablation, RT= radiotherapy, yr.(s), year(s).

Case report reference	Neoadjuvant treatment	Patient characteristics	Short-term response	Long-term response
Angiosarcoma of the breast				
Primary angiosarcoma of the breast				
[21]	4 cycles of ifosfamide, vincristine and dactinomycin	1 pt.	Tumor reduction of 50%	Disease free after 2 yrs. of FU
[22]	Arterial injection with cyclophosphamide and 5-FU	1 pt.	No short-term FU data available	Disease free after 15 months of FU
[23]	Gemcitabine and docetaxel	1 pt.	pCR	No evidence of recurrence 20 months after the initial diagnosis
[24]	Gemcitabine and docetaxel	1 pt.	pCR	Disease free after 2 yrs. of FU
[25]	4 cycles of cisplatin, doxorubicin and thalidomide, followed by paclitaxel, cisplatin and thalidomide	1 pt.	pCR in the breast and axillary lymph nodes	No recurrence 6 months after the initial diagnosis
Radiation induced angiosarcoma of the breast				

[26]	4 cycles of gemcitabine and docetaxel	1 pt.	Clinical improvement after 2 cycles, near CR on MRI after 4 cycles	No FU data available
[27]	3 cycles of gemcitabine and docetaxel	1 pt.	Minimal residual disease in resected tissue	Disease free after 9 months of FU
[28]	8 cycles of carboplatin and gemcitabine	1 pt.	Improvement of local condition of the breast	No recurrence 1 yr. after the surgery
Angiosarcoma of the face and scalp				
[29]	3-4 cycles of bevacizumab and RT 50 Gy	2 pts with AS of the face	pCR	Disease free after 8.5 (pt. 1) and 26 months (pt. 2) of FU
[30]	5 cycles of paclitaxel Thereafter 5x PDT	1 pt. with AS of the scalp	No metastasis, no improvement of skin lesions Improvement of all skin lesions	Disease free after 6 months of FU
[31]	1 cycle of cisplatin, docetaxel and 5-FU	1 pt. with radiation induced AS of the face	Decreased tumor size from 35x21mm to 19x13mm on MRI	Lung metastasis after surgery. Progressive metastasis after AT
[32]	Cyclophosphamide, vincristine, doxorubicin and dacarbazine Doxorubicin, ifosfamide and dacarbazine	3 pts with post-irradiation AS (AS location not specified)	PR 1/3 pts	No FU data available
Cardiac angiosarcoma				
[33]	Doxorubicin, dacarbazine, ifosfamide and mesna followed by RT 2600 cGy for 1 month	1 pt.	Not specified	Disease free after 33 months of FU
[34]	3 cycles of doxorubicin and dacarbazine	1 pt.	Tumor became operable	Disease free after 2 yrs. of FU
Other				

[35]	3 cycles paclitaxel	1 pt. with AS of the spleen	PR after 3 cycles on CT	No recurrence 14 months after start of treatment
[36]	Vincristine, cyclophosphamide and actinomycin	1 pt. with calvarial AS	PD	Disease free after 3 yrs. of FU
[37]	2 cycles of ifosfamide, doxorubicin, mitomycin, cisplatin and mesna Followed by 50 Gy and 2 cycles mitomycin, doxorubicin and cisplatin	1 pt. with AS of seminal vesicle	After 2 cycles of NAC decreased tumor size from 5.6×5.1 to 4.3×4.0 cm No significant changes after RT	Disease free after 6 yrs. of FU
[38]	1 cycle of taxol, followed by 3 cycles of gemcitabine	1 pt. with epithelioid AS of the thyroid	<10% viable tumor cells left in surgical specimen	Disease free after 70 months of FU

Retrospective cohort studies

UV-induced angiosarcoma of the face and scalp

One of the cohort studies focused on patients with UV-induced angiosarcoma of the face and scalp.

In the cohort published by Guadagnolo *et al.*, 70 patients with angiosarcoma of the face and scalp were included of whom 44 patients (63%) received chemotherapy (33 neoadjuvant and 11 adjuvant). The addition of chemotherapy to the standard treatment was independent of the size of the tumor and most patients received the combination of gemcitabine and docetaxel or paclitaxel single agent. From the 33 patients with neoadjuvant chemotherapy, eleven patients showed a clinical CR (33%), eighteen patients a PR (55%), two patients a SD (6%) and two patients PD (6%). Nine of the patients treated with neoadjuvant chemotherapy also received adjuvant chemotherapy (27%) [17]. In this study neither the status of the resection margins, nor the addition of chemotherapy had an influence on the OS or DSS when compared to the patients who did not receive chemotherapy. Neoadjuvant chemotherapy slightly improved the 5-year distant metastases free survival (38% (n=33) vs. 69% (n=37), p= 0.06), but did not improve the local control after surgery [17].

In summary, based on this very limited sample size with an unknown patient selection for neoadjuvant chemotherapy, no conclusions can be drawn on the effect of neoadjuvant chemotherapy on the local and distant control rate of UV-induced angiosarcoma of the face and scalp. However, response rates were relatively high with only 6% PD during chemotherapy.

Cutaneous angiosarcoma

While the current European guideline [16] does not give strict guidance in the use of neoadjuvant chemotherapy, it was already implemented as standard of care for cutaneous angiosarcoma in the Roswell Park Center since 2008 [13]. Neoadjuvant chemotherapy is used to treat occult micrometastases and to identify patients who would not benefit from a potentially morbid surgery. Patients who develop metastasis or with rapid PD during neoadjuvant chemotherapy, would be excluded from extensive surgery. Oxenberg *et al.* retrospectively compared data from patients with neoadjuvant chemotherapy and surgery with surgery alone [13]. They included 25 patients treated between 1996-2012 with cutaneous angiosarcoma at different locations, including breast and head and neck. From these patients, thirteen patients had a primary resection and twelve patients were treated more recently and started with neoadjuvant chemotherapy of whom eventually ten patients underwent surgery. Two patients, who developed distant metastases during neoadjuvant chemotherapy, were excluded from further comparisons. The response and outcome analyses were performed for the two subgroups as total (surgery alone (n=13) vs. neoadjuvant chemotherapy and surgery (n=10)), despite the heterogeneity of tumor localizations and the difference in follow-up time within the groups. Two different chemotherapeutic regimens were given: paclitaxel (n=6) or gemcitabine plus docetaxel (n=4). There were no differences in resection margins or type of wound closure between the two groups. Thirty percent of

the neoadjuvant chemotherapy cohort had a pathologic CR (pCR), however, neoadjuvant chemotherapy did not improve the local RFS, distant DSS, DSS or OS [13]. On the other hand, delay in surgery due to neoadjuvant chemotherapy did not negatively influence the outcome of these patients either.

Sinnamon *et al.* searched a large national database and included 821 patients with localized cutaneous and soft tissue angiosarcoma, who underwent surgery [18]. They excluded patients who died within 90 days after surgery, which could have confounded the results. Of the 821 patients, 26% was located in the head and neck region. Overall, only 38 patients (5%) received neoadjuvant chemotherapy, but the rationale for choosing neoadjuvant treatment in these patients was not specified. Nevertheless, both neoadjuvant (median OS 3.1 years, n=38) and adjuvant chemotherapy (median OS 3.8 years, n=128) did not improve the median OS compared to the median OS of patients without chemotherapy (3.4 years, n=655) [18]. Of note, the results could be biased, because patients with a worse prognosis, caused by larger tumors or tumors which are located in areas which are difficult to operate, are more likely to receive neoadjuvant treatment. Furthermore, no information about the chosen regimen was provided, which makes it complicated to interpret these results, because the type of chemotherapy could also affect the outcome of patients. The large number of patients in this cohort created the opportunity to identify factors associated with poor OS using Cox proportional hazards modeling. Factors significantly associated with poor survival, with descending hazard ratio (HR), were tumor size >7 cm (HR 2.37), age >70 years (HR 2.02), Afro-American race (HR 1.92), tumor size 3-7 cm (HR 1.64), positive resection margins (microscopic HR 1.59, macroscopic HR 3.38), grade 3 tumor (HR 1.52) and head and neck as primary localization (HR 1.44) [18].

To conclude, both cohort studies investigated the effect of neoadjuvant chemotherapy in patients with non-metastatic cutaneous or soft tissue angiosarcoma and found no survival benefit, but also no dismal effects of delaying the resection.

Cardiac angiosarcomas

Two of the retrospective cohort studies investigated cardiac sarcomas. Li *et al.* focused on the survival after a heart transplantation as an uncommon treatment of unresectable non-metastatic cardiac sarcomas in six cases from their own institute and 40 patients from the literature [19]. Among the 46 patients receiving heart transplantation for primary cardiac sarcoma, angiosarcoma was the most common histologic subtype (n=14, 30%). The 46 patients with a heart transplantation were compared to seven patients with unresectable, non-metastatic cardiac sarcomas of the same institute who only received palliative treatment (systemic therapy or radiotherapy), due to patient choice or unavailability of a donor heart [19]. They found that the survival after heart transplantation was worse for angiosarcomas than other cardiac sarcomas (9 vs. 36 months, p=0.002) and the survival after heart transplantation was comparable to patients receiving palliative systemic treatment only (9 vs. 8 months, p=0.912) [19]. Furthermore, neoadjuvant as well as adjuvant chemotherapy did not improve the survival for all cardiac sarcoma patients (15 vs. 18

months, p=0.210, and 15 vs. 26 months, p=0.088, respectively) [19]. However, the rationale for the addition of neoadjuvant chemotherapy was not given in the manuscript.

Abu Saleh *et al.* have previously shown that in the treatment of cardiac sarcomas R0 resection margins resulted in better OS, but this was not easily achieved [20]. They hypothesize that neoadjuvant chemotherapy could result in debulking of the tumor and therefore could aid in achieving negative margins during surgery. They included 44 cardiac sarcoma patients of whom the majority had angiosarcoma (n=30, 68%). As part of a clinical trial to investigate the effect of neoadjuvant chemotherapy on the survival, 32 patients received neoadjuvant chemotherapy, of which 24 (80%) patients with angiosarcoma. The demographic characteristics were comparable between the group who received neoadjuvant chemotherapy and the group who received no chemotherapy. However, stage at start of treatment differed between the groups, 63% of the patients in the neoadjuvant group had distant metastases and only 33% in the group treated without chemotherapy (p=0.082). The first line neoadjuvant treatment of the sarcoma patients consisted of doxorubicin plus ifosfamide and the second line consisted of gemcitabine plus docetaxel. Both patients with local and limited metastasized disease were included, if they were considered eligible for surgery. An R0 resection resulted in a five times longer median survival and neoadjuvant chemotherapy doubled the R0 resection rate (24% vs 61%, p=0.03) [20]. The 30-day mortality rate was lower in the group who received neoadjuvant chemotherapy but not significantly (3 vs 8%, p=0.476) and there was no difference in 30-day postoperative complications [20].

Based on these two retrospective cohort studies, the addition of neoadjuvant chemotherapy to resection of the tumor could be a preferable therapeutic approach with a good safety profile and an improved R0 resection rate in a selective patient group of operable cardiac angiosarcoma. In inoperable non-metastatic cardiac sarcoma patients, a heart transplantation with or without neoadjuvant or adjuvant chemotherapy does not result in a survival benefit.

Other

The group of Fayette *et al.* looked into a dataset of 164 patients with all the different histological angiosarcoma subtypes [14]. From these patients, data regarding systemic treatment was available of 144 patients. Seventeen patients received chemotherapy after R2 resection or for inoperable disease, with a 59% response rate (18% CR, 41% PR), 12% SD and 29% PD during treatment. The demographic characteristics of the different treatment groups were not compared in this study. Treatment regimens were either doxorubicin alone, ifosfamide alone or a combination of doxorubicin with ifosfamide. However, chemotherapy did not result in a significant difference in OS or PFS [14]. Smaller tumor size (<5 cm), histological grade (low and no necrosis) and R0 resections were associated with a better OS [14]. Neither the rationale for the addition of neoadjuvant chemotherapy to standard treatment, nor the precise response rate of the patients who received neoadjuvant chemotherapy was provided [14]. Furthermore,

another chemotherapy regimen could have resulted in more activity as most current studies use a taxane based regimen.

Conclusions retrospective cohort studies

The cohort studies consisted of very heterogeneous patient groups, treated with various regimens. Patient selection for neoadjuvant chemotherapy was often not substantiated, allowing potential selection bias. Therefore, no definite conclusions regarding outcome benefit for these patients can be drawn based upon this data.

However, within these cohort studies, with heterogeneous treatment regimens and follow-up periods, the response rate (PR or CR) to neoadjuvant chemotherapy was extremely high with 88-93% for face and scalp angiosarcoma [13,14,17–20].

Case reports

In total, eighteen case reports describing 21 patients were previously published in the literature. In this review, we will discuss the cases per tumor localization (table 1b). Potential publication bias should be considered.

Angiosarcoma of the breast

Eight cases of angiosarcoma of the breast have been reported of which three especially describe radiation induced angiosarcoma [23–28]. The patients were treated with a variety of chemotherapy schedules and all patients showed a response (CR or PR) to neoadjuvant chemotherapy.

From the five patients with primary angiosarcoma of the breast [18-20,26,27], one patient had a PR (50% tumor reduction after ifosfamide/vincristine/dactinomycin) [21] and three patients had a pCR: two after treatment with gemcitabine and docetaxel and one after cisplatin/doxorubicin/paclitaxel given concurrently with thalidomide [18,19,27]. No short term follow-up data on tumor reduction was available for the fifth case, but the patient was disease free 15 months after neoadjuvant therapy with an injection of cyclophosphamide/5-FU into the artery that supplied the tumor and surgery [22]. None of the patients had recurrent disease during the reported follow-up period (range 0.5-2 years).

Of the patients with radiation induced angiosarcoma [26–28], all patients were treated with neoadjuvant gemcitabine, two combined with docetaxel [27,28] and one combined with carboplatin [26]. Each patient showed clinical improvement after chemotherapy and the two patients with available follow-up data were disease free after 9 months and 1 year, respectively [27,28].

In these cases, angiosarcoma of the breast was quite sensitive to chemotherapy with clinical responses in all patients. All patients were disease free after a follow-up of 6-24 months. Of note, non-responding patients are generally not overrepresented in case reports.

Angiosarcoma of the face and scalp

Three case reports (four patients) elaborate on neoadjuvant systemic therapy in UV-induced cutaneous angiosarcoma of the face and scalp [29–31]. Two patients had a pCR after treatment with bevacizumab and radiotherapy. After 8 and 26 months of follow-up the patients were still disease free [29]. One patient did not show a response after five cycles of paclitaxel, but had a remarkable response on photodynamic therapy and was still recurrence free after 6 months [30]. The third case report describes a patient who had a decline of the tumor size after treatment with cisplatin plus docetaxel plus 5-FU, but unfortunately developed distant metastases shortly after the surgery [31].

In summary, three out of four patients with UV-induced angiosarcoma of the face and scalp had a response to neoadjuvant chemotherapy. Three patients were disease free after 6-26 months, one had metastatic disease shortly after surgery. Finally, the study from des Guetz *et al.* [32] describes three patients with radiotherapy associated angiosarcoma who were treated with neoadjuvant chemotherapy. One of these patients had a PR after neoadjuvant chemotherapy. Which chemotherapy regimen this patient received, was not specified.

Cardiac angiosarcomas

Two case reports describe patients with cardiac angiosarcoma [33,34]. All patients received doxorubicin based regimens to enhance the resectability of the tumors followed by resection of the tumor in one patient [34] and a heart transplantation in another patient [33]. In one patient neoadjuvant treatment was used to downstage the disease to enable surgery [34]. All patients showed a positive response to neoadjuvant chemotherapy and are disease free after a follow-up period of 24-33 months [33].

Angiosarcoma with other origin

The remaining case reports describe four cases with a histologically proven angiosarcoma with rare sites of origin:, one in the spleen [35], one in the calvarial space [36], one in the seminal vesicle, [37] and one in the thyroid [38]. These four patients received a variety of neoadjuvant therapies, which makes it difficult to interpret the impact of these separate cases for a general treatment advise. Almost all patients showed a response to chemotherapy and all patients showed long term disease control after surgery [35–38].

Discussion

Given the often dismal prognosis of angiosarcoma, neoadjuvant systemic therapy is increasingly being considered as a valid treatment option to downsize the tumor, facilitating adequate surgical resection, but also to evaluate tumor biology to prevent unnecessary extensive surgery in case of early metastases, and prolong survival. However, literature discussing neoadjuvant strategies is limited, as we show in this systematic review. The cohort studies (table 1a) consisted of heterogeneous patient

groups with low patient numbers, and included both- prognostically different- primary and secondary angiosarcomas, [7] patients treated with various treatment regimens and with different follow-up periods. Neoadjuvant chemotherapy was more often added to the standard treatment of recently diagnosed patients. Patient selection for neoadjuvant chemotherapy was often not substantiated and therefore, there will almost certainly have been a selection bias. And lastly, with the improvement of current histological diagnostics, some of the more previously diagnosed angiosarcomas are probably not real angiosarcomas, but other vascular tumors [7]. The study of Weidema *et al.* even showed that 16% of the angiosarcoma patients was wrongly classified as angiosarcoma after reevaluation of the histological material, however with a clear improvement since the introduction of molecular diagnostics [7]. Therefore, no definite conclusions can be drawn based on these data. Nevertheless, within this retrospective cohort studies with heterogeneous treatment regimens, the response rate (PR or CR) after neoadjuvant chemotherapy was very high for face and scalp angiosarcoma. No survival benefits were seen after neoadjuvant chemotherapy, although, in fact, this can only be assessed properly in randomised trials, which are lacking.

Because of the retrospective nature of the studies, these results should be interpreted with caution. Patient numbers are low and a wide diversity of chemotherapeutic regimens were investigated within different tumor sites of origin. Besides, patients with locally, primary and recurrent disease were all included, despite the influence of these characteristics on the outcome of angiosarcoma patients. Theoretically, patients with recurrent disease, with lymph node dissections or with inoperable disease might benefit more from neoadjuvant chemotherapy than patients with primary angiosarcoma. Most importantly, there is an enormous selection bias in patients receiving neoadjuvant chemotherapy, since neoadjuvant chemotherapy is not standard of care in most hospitals. Patients with more advanced disease and high-risk disease are probably selected for neoadjuvant chemotherapy, which impacts the interpretation of survival comparisons with smaller, primary resectable, tumors. Selection bias could also have occurred the other way around, since mostly younger and more fit patients are selected for neoadjuvant chemotherapy, because they can manage the treatment toxicity better, which would result in an overestimation of overall survival. Unfortunately, in most studies, the rationale for patient selection was not discussed and it is therefore extremely difficult to draw any conclusions on the effect of chemotherapy on survival.

Additionally, the type of angiosarcoma influences the outcome. For instance, patients with cardiac or visceral angiosarcoma have a worse prognosis compared to patients with cutaneous angiosarcoma [7,19,20,27]. Patients with cutaneous UV-induced angiosarcoma have a relatively better survival, despite the challenge in getting

clear surgical margins [18]. Furthermore, these patients often have multi-satellite disease [17]. In patients with angiosarcoma of the scalp, the aim of neoadjuvant chemotherapy could primarily be to achieve less (mutilating) surgeries rather than achieving prolonged survival. Prognostic factors which were independently correlated with a worse prognosis were positive resection margins, primary location on the face or scalp, tumor size (>5.0 cm), grade 3 histology, multi-satellite disease, older age (>70 years), primary angiosarcoma, Afro-American ethnicity, metastatic disease and worse performance status [7,13–15,18,20]. All these prognostic factors should be taken into account to make a clean interpretation of the effect of the addition of neoadjuvant treatment to the standard of care.

Another important conclusion, also highlighted by Oxenberg *et al.*, is that any delay in surgery caused by neoadjuvant chemotherapy did not seem to influence the outcome, since there was no difference in outcome between patients who received neoadjuvant chemotherapy and patients who did not, despite the fact that two patients were progressive under chemotherapy and did not receive the planned surgery [13]. Additionally, neoadjuvant chemotherapy could offer additional time to observe the tumor biology and identify these progressive patients who would not benefit from aggressive surgery [13]. Furthermore, we did not find any studies reporting a worse outcome with the addition of neoadjuvant chemotherapy. Therefore, the addition of a neoadjuvant treatment to standard of care could be a safe and individualized option for a selected group of patients [13,20].

Lastly, it is unclear which chemotherapy regimen is giving the best results in angiosarcoma in general. Despite excellent short-term responses, the benefit for the long-term outcome is debatable. Taxanes, doxorubicin and gemcitabine regimens all report responses, but alternatives may be considered. For example, because of the high expression of beta-receptors on vascular tumors the addition of the beta-blocker propranolol to chemotherapy based regimens might be beneficial according to literature [39–41]. Furthermore, newer drugs such as checkpoint inhibitors, have shown relatively good responses in especially the UV-induced angiosarcoma, making this a potential drug to use in the neoadjuvant and metastatic setting [1,41–44]. In particular in elderly with cutaneous angiosarcoma paclitaxel may give durable responses [45]. Currently there is one recruiting study in which paclitaxel is combined with chemoradiation as induction treatment of cutaneous angiosarcoma (NCT03921008).

To summarize, there are several limitations of this review which are important for the interpretation of the results. Current literature only consisted of retrospective studies of farheterogeneous patient populations with low patient numbers, treated with various regimens and lacking the rationale for treatment choice or evaluation of possible confounders in treatment response. Considering these limitations of the angiosarcoma studies so far and the challenges in performing a randomized controlled trial in a rare tumor type, an international registry with data on angiosarcoma could be a very valuable source of information. An easily accessible registry could help to develop international treatment guidelines, identify new treatment targets and elucidate angiosarcoma characteristics. Recently in the US, a project was set up to

collect angiosarcoma patient data. Patients are approached via social media and patient advocacy groups and give their consent online, making it a very innovative patient-partnered approach [46]. Expansion of this kind of databases to other countries, would help in the design and execution of new randomized trials, to increase patient numbers, and provide internationally accepted treatment guidelines. But the challenge of data protection is certainly something that needs to be addressed.

Materials and Methods

A search was performed in Ovid Medline, Ovid Embase, Cochrane library and Scopus with thesaurus terms and words in title, abstract and (author) keywords. We searched for angiosarcoma, hemangiosarcoma and lymphangiosarcoma in combination with terms for 'neoadjuvant therapy', 'preoperative therapy', 'targeted therapy' and 'immunotherapy'. The searches were performed on 25 October 2019. We applied no limits in publication date. Additional articles were included using citation snowballing. Selection of relevant studies was performed independently by two authors. Conflicts in the selection of relevant articles were resolved by discussion. All studies that evaluated the effect of neoadjuvant systemic therapy in the treatment of primary, secondary or recurrent angiosarcoma on the resection margins and the long term survival were eligible. A quality assessment was performed using the Newcastle Ottawa scale for cohort studies (*supplemental table 1*, *available online at Cancers*) [47].

In this systematic review the terms complete response (CR), partial response (PR) and progressive disease (PD) refer to the terms as defined in the Response Evaluation Criteria in Solid Tumors (RECIST) [48] and were mostly measured clinically. Outcome was given in terms of disease free interval (DFI), progression free survival (PFS), disease specific survival (DSS) and overall survival (OS).

Conclusions

Unfortunately, no definitive conclusions can be drawn regarding the outcome benefit of neoadjuvant chemotherapy in patients with angiosarcoma based on the current literature. All available studies were retrospective with heterogeneous, small patient groups and diverse treatment regimens with the inherent limitations. Keeping these limitations in mind, however, the retrospective cohort studies and case reports suggest that angiosarcoma is relatively sensitive to chemotherapy (response rate of 88-93% in patients with angiosarcoma of face and scalp). Neoadjuvant chemotherapy could therefore probably be used to downsize the tumor. This downsizing could result in more resections with curative intend, less mutilating resections and a higher R0 resection rate (an increase of 5-14% of all angiosarcomas to even 50% of cardiac angiosarcomas). The studies show no clear survival benefit. Nevertheless, there is an urgent need for more studies addressing the role of neoadjuvant systemic therapy in

angiosarcoma and an international angiosarcoma registry could help to develop guidelines.

Recommendations based on this review of the literature:

- Neoadjuvant chemotherapy could be considered to downsize the tumor, since this could lead to less mutilating resections and a higher R0 resection rate.
- There is no survival benefit, but also no evidence of detriment of neoadjuvant chemotherapy.
- There is currentlyno evidence of the best possible chemotherapy regimen and apart from age of the patient, also the subtype may help define the treatment choice. In particularly for UV-exposed scalp angiosarcoma in elderly, paclitaxel is generally well tolerated and more recently also checkpoint inhibitors are showing interesting responses.
- An international angiosarcoma registry should be set up to collect all available data on angiosarcoma patients and help to develop guidelines.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. WvdGraaf: research grant from Novartis and fees for advisory boards from Bayer and Springworks, all to the Institute. JH Beijnen holds a patent on pharmaceutical taxane formulations and is (part time) employee and stock holder of Modra Pharmaceuticals, a small spin out company developing clinically oral taxane formulations. NSteeghs provided consultation or attended advisory boards for Boehringer Ingelheim, Ellipses Pharma.; received research grants for the institute from AB Science, Abbvie, Actuate Therapeutics, Amgen, Array, AstraZeneca/MedImmune, Bayer, Blueprint Medicines, Boehringer Ingelheim, Bristol-Myers Squibb, Cantargia, Cytovation, Deciphera, Genentech/Roche, GlaxoSmithKline, Incyte, InteRNA, Lilly, Merck Sharp & Dohme, Merus, Novartis, Pfizer, Pierre Fabre, Roche, Sanofi, Taiho, Takeda (outside the submitted work). This all is not related to this manuscript.

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CHAPTER 6.2

PropAngio study protocol: a neoadjuvant trial on the efficacy of propranolol monotherapy in cutaneous angiosarcoma. A proof of principle study.

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Published online in BMJ open 2020, September 10.
Study Protocol.

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Abstract

Introduction: Angiosarcoma is a rare and aggressive malignancy with a high metastatic potential and recurrence rate. Despite optimal treatment with surgery, with or without radiation, the prognosis remains poor and, therefore, new treatment strategies are warranted. Recently, propranolol has effectively been repurposed for the treatment of infantile hemangioma. Propranolol is a β3-sparing antagonist of the β-adrenergic receptor. In infantile hemangioma, the β1, β2 and β3-receptors are highly expressed. Angiosarcoma has several similarities with hemangioma, including its high β-adrenergic receptor expression and the supposedly important role of VEGF in malignant growth. As a result, propranolol has been administered small-scale in individual angiosarcoma cases with promising results. The precise effect of propranolol, however, is not yet established.

Methods and analysis: The goal of this neoadjuvant window of opportunity study is to prospectively evaluate the activity of propranolol monotherapy in patients with cutaneous angiosarcoma. The neoadjuvant setting provides a good opportunity to rapidly evaluate both the clinical response and histological response, without a significant delay in standard anti-cancer treatment. Fourteen patients with primary, recurrent or metastatic cutaneous angiosarcoma will be included. Propranolol will be administered orally in an escalating dose during three to six weeks, before the initiation of standard treatment. The primary endpoint is clinical response according to RECIST, as measured on consecutive coloured photographs or CT/MRI. The histological response will be determined as secondary endpoint, comparing the difference in proliferation index before and after propranolol by measuring the change in immunohistochemistry staining of Ki-67. The study will be considered positive when at least 3 patients have a response to propranolol.

Ethics and dissemination: Ethical approval was obtained from the Medical Ethical Committee of the Netherlands Cancer Institute. Independent of the outcome, results of this study will be shared and submitted for publication in an international peer-reviewed journal.

Trial registration number: Registry through the Netherlands Trial Register (Trial no. NL8118).

Strengths and limitations of this study

- The neoadjuvant setting provides the opportunity to evaluate the antitumor response of propranolol monotherapy without delaying the standard treatment.
- The propranolol dose will be escalated to optimize the safety profile of the treatment.
- As it is a window of opportunity study, the study duration will be relatively short.
- A limitation of the current design (proof of principle study), is the absence of randomisation.

Introduction

Angiosarcoma is a rare and aggressive malignancy with a high metastatic potential. The estimated incidence of angiosarcoma is 0.4 per million patients per year, making it a very rare disease.[1] The standard of care for localised angiosarcoma is currently complete surgical resection with or without radiation. Unfortunately, despite the current standard of care, only 60% of patients with localized disease survive for more than five years.[2] Physicians and researchers are, therefore, in urgent need to find better treatment options for these patients.

Various additional drugs for systemic treatment have been investigated before.[2–4] Although the role of (neo)adjuvant chemotherapy remains controversial for localised disease, neoadjuvant chemotherapy is often administered for locally advanced angiosarcoma.[3,5–8] Several cytotoxic drugs, including anthracyclines, taxanes and gemcitabine, have shown activity in angiosarcoma in the locally advanced and metastatic setting, with overall response rates varying from 17 to 89%.[2–4] However, for the treatment of resectable angiosarcoma, none of the previous studies show a prolonged disease-free survival or overall survival.[3,5–10] Improved treatment in the neoadjuvant setting might reduce the local and distant recurrence rates by treating micrometastases at an early stage and by improving the resection margins, potentially leading to higher survival rates. As a result, new drugs are urgently needed to prolong the survival.

Propranolol hydrochloride, a synthetic β3-sparing-adrenergic receptor antagonist, was registered by the Food and Drug Agency (FDA) decades ago for the treatment of hypertension. Drug repurposing is a drug development strategy focused on the reuse of existing drugs for new medical indications. Recently, propranolol has been repurposed and is now used in the treatment of infantile hemangioma. Infantile hemangioma is a benign vascular tumour and propranolol dosed 3 mg/kg led to a complete to near complete resolution in approximately 88% of the treated infants with

infantile hemangioma.[11,12] The pharmacological effects of propranolol in infantile hemangioma are presumed to cause vasoconstriction, a decreased expression of vascular growth endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), inhibition of migration and proliferation of tumour cells and induction of apoptosis of endothelial cells.[12–16] Angiosarcoma have several similarities with infantile hemangioma, including its high β-adrenergic receptor expression and the suggested important role of VEGF in malignant growth.[14,17,18]

Several small case reports and case series have confirmed the idea that propranolol could be repurposed to treat angiosarcoma. In these case reports, patients with locally advanced or metastatic angiosarcoma were treated with propranolol, in combination with various chemotherapy regimens, including combination therapy with cyclophosphamide, etoposide, paclitaxel and vinblastine-based chemotherapy. The dose of propranolol in combination therapy varied between 80 to 120 mg per day.[20–25] In one case there was a response after 1 week of propranolol monotherapy 40 mg twice a day (BID).[22] These doses of propranolol are much lower than the standard maintenance dose of 160-320 mg daily for patients with hypertension.[25] Furthermore, there was a reduction in the proliferative index of 34%, stabilization of tumour growth and less necrosis.[22] Additionally, one case described a patient with metastatic cardiac angiosarcoma who showed a long term response (>12 months) to propranolol monotherapy, while the mean survival time is only four months.[25]

Since literature regarding the activity and mode of action of propranolol as a single agent for angiosarcoma is scarce, our aim is to evaluate the activity of propranolol monotherapy in patients with primary, recurrent or metastatic cutaneous angiosarcoma before they proceed to their standard anti-cancer treatment. The neoadjuvant setting provides a good opportunity to rapidly evaluate both the clinical and histological response, without delaying the standard anti-cancer treatment. Additional advantages of propranolol therapy would be the ease of use and the relatively mild toxicity profile. If this study turns out to be positive, further (randomized) clinical trials are thereby substantiated and highly recommended.

Methods and analysis

Aim and objectives

The aim of this study is to investigate the effect of neoadjuvant propranolol monotherapy in patients with primary, recurrent or metastatic cutaneous angiosarcoma, before they proceed to their standard anti-cancer treatment (e.g. isolated limb perfusion, chemotherapy, targeted therapy, surgical resection or radiotherapy). The primary objective is to determine the clinical response of

propranolol monotherapy and the histologic response will be evaluated as secondary objective.

Study design and study treatment

This is a prospective proof of principle study with neoadjuvant propranolol monotherapy in cutaneous angiosarcoma patients. We will use the neoadjuvant window as an opportunity to explore the activity of propranolol monotherapy, without delaying the standard treatment. The duration of treatment will be three to six weeks. In this single arm trial, angiosarcoma patients will be treated with propranolol monotherapy in an intrapatient escalating dose, which will be adjusted to the tolerability of propranolol. The treatment plan of propranolol is provided in *Table 1*. The treatment plan was designed based on doses used in previous literature [15,21,24,25] and not exceeding the maximum maintenance dose of 320 mg/day for the registered indication hypertension (maximum daily dose in our study 240 mg/day).[25] In case of hypotension (blood pressure <90/60 mmHg) or bradycardia (heart rate <55 bpm), or symptoms of bradycardia or hypotension (dizziness, syncope) the dose will be reduced to the previous dose level. In case of serious bradycardia (heart rate <50 bpm), the treatment will be stopped until an acceptable heart rate (>55 bpm) is reached and propranolol will be restarted in the dose of the previous level.

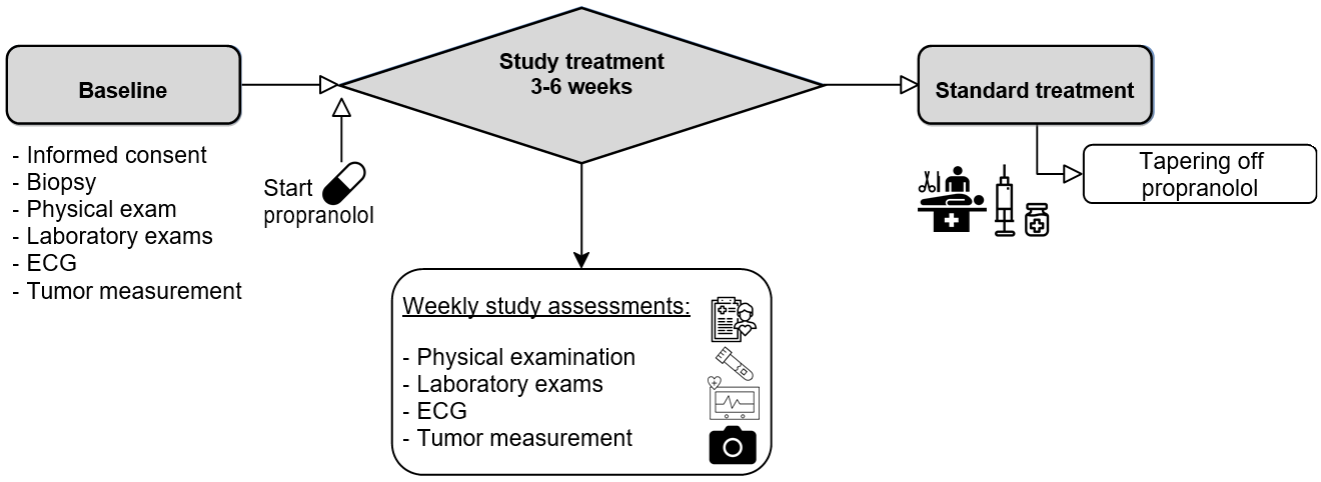
Table 1. Propranolol treatment plan.

Dose escalation scheme		
Period	**Dose level**	**Dose**
Day 1 – Day 7	1*	2x/day 40 mg
Day 8 – Day 14	2*	2x/day 80 mg
Day 15 – Day of surgery or biopsy	3*	3x/day 80 mg
Tapering off scheme after surgery/biopsy		
Period	**Dose**	
Day 1 - Day 7	2x/day 80 mg	
Day 8 - Day 14	2x/day 40 mg	

*All patients start on day 1 with dose level 1.

The tolerability will be assessed during weekly visits in the outpatient clinic (*figure 1*). Each visit consists of a physical examination, blood draw for safety assessment (hematology, hepatic- and renal function), vital signs, electrocardiogram (ECG), toxicity assessment, concomitant medication registration and tumour response assessment. After the study treatment, a biopsy will be obtained to evaluate the histologic response to propranolol monotherapy. Propranolol will be tapered off after the biopsy, to prevent withdrawal symptoms (*table 1*).

Figure 1. Study assessments. Figure 1 gives an overview of the study assessments, which are planned at baseline, during study treatment or when the standard treatment is initiated.



Patient selection

The study population consists of patients with primary, recurrent or metastatic cutaneous angiosarcoma, including angiosarcoma of the breast (radiation induced). Only patients with cutaneous angiosarcoma can participate, since these tumours are easily measurable on coloured photographs for clinical response evaluation. Patients are eligible if they are at least 18 years old; have a good performance status (world health organization (WHO-PS) of 0-2); have an adequate blood count, kidney and renal function; have a window of at least three weeks between their diagnosis and the start of the standard anti-cancer treatment and have evaluable disease according to the Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 criteria.[27] Patients with primary visceral angiosarcoma, contraindications for β-blockade therapy or current treatment with β-blockade therapy (both selective and non-selective β-blockade therapy) or other anti-cancer treatment are excluded.

Sample size calculation

An exact single-stage phase II design will be used with a one-sided significance level α of 0.05 and a power of 80%. The maximum response rate that would be of no interest was assumed to be 5% and the minimum required response rate 30%. A total of 14 patients will be included in the study. If 3 or more out of these 14 patients have a response as defined in the study endpoints, the study is considered positive.

Study endpoints

Primary endpoint

The clinical response will be determined according to RECIST 1.1 criteria (PD is >20% increase in size, PR is >30% decrease in size, SD is in between while CR is no

measurable disease).[27] A response is defined as CR, PR, or SD with an improvement in clinical characteristics, like thickness, erythema, necrosis or edema of the inflicted area. Documentation will be performed with colour photography, including a ruler to measure the size of the lesion. Radiologic assessment will only be done if the patient has radiologic evaluable disease at the beginning of the study treatment. If the study turns out to be positive, this treatment modality is highly interesting and should be tested further in a randomized trial.

Secondary endpoint

The histologic response on propranolol treatment is defined as difference in proliferation index. This will be assessed by measuring the change in immunohistochemistry staining of Ki-67 and the tumour activity between the post-treatment biopsy (obtained during surgery if applicable) and the diagnostic biopsy before the study treatment. A decrease of >30% of the Ki-67 staining will be considered as a positive histologic response.

Exploratory endpoints

To obtain additional data regarding the primary objectives, the percentage of adrenergic receptors (β1-AR, β2-AR, β3-AR)in the pre-treatment biopsies will be measured with immunohistochemically staining and the correlation with the anti-tumour response of the angiosarcoma patients will be investigated.[13,22] With this correlation analysis, the predictive value of adrenergic receptor expression in tumour tissue on the anti-tumour response will be assessed. Finally, we will compare the PET response before start of treatment and at the end of propranolol treatment.

Study logistics

Patient recruitment and study duration

Treating physicians will identify patients as possible candidates and inform patients about the study. If patients agree to participate and fulfil the selection criteria, patients will be included during an outpatient clinic visit. As this is a monocenter study, all patients will be included in the Netherlands Cancer Institute (NKI). Approximately 20 new angiosarcoma patients are seen in the NKI yearly. As a result, the expected duration of the study is two years. Enrolment started on 27 December 2019.

Safety assessments

All adverse events will be recorded in the electronic case report forms (eCRF) during the weekly outpatient clinic visits. We will perform extra blood draws, ECGs and measurements of the vital signs during these visits for safety assessments. The

recording of the adverse events will be done according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (CTCAE) version 5.0.

Data management

The original results will also be recorded in the eCRF by the investigators of the study. The data entry will be supervised by the Clinical Research Monitor.

Study monitoring

Monitoring of the study will be performed according to ICH GCP by the Clinical Research Monitor of the NKI or the person to whom the monitoring tasks have been delegated. Amongst others the following will be reviewed: compliance with the protocol, ICH GCP and all applicable regulatory requirements; consent procedures, including date of consent and signatures; study progress; (Serious) Adverse Events; completion of the (e)CRFs and verification of data against the source data; and storage, dispensing and accountability of study medication.

The Medical Ethical Committee of the NKI will review the study every year throughout the complete study duration. During this review, the committee will focus on monitoring of the safety of patients and evaluate the balance between the efficacy and the harmfulness of propranolol.

Termination of the study

An interim analysis is planned after the treatment of seven patients. If there are already three or more responses at this time point, the study will be stopped and stated positive. Otherwise an additional seven patients will be included. Results of the study will be shared and be submitted for publication in an international peer-reviewed journal.

Patient and public involvement statement

The trial protocol and other trial documents were developed in collaboration with the Dutch sarcoma patient advocacy group. They evaluated the specific patient need for this trial. They fully support this trial and the concept of exploring drug-repurposing strategies to improve outcome in sarcoma. The patient advocacy group will be informed about the progress of the study and the study time lines.

The study is funded by a Belgian non-profit organisation: the Anticancer Fund. Their mission is to complement current cancer care with patient-first thinking and a focus on evidence-based potential for new treatments. Financially, the Anticancer Fund is completely dependent on donations and private funding. The Anticancer Fund supports diverse clinical trials, mainly in under-prioritised treatment groups (such as in

rare tumours), with non-conventional therapies and repurposed drugs. The trial was registered in the Netherlands Trial Register (NL71090.031.19).

Ethics and dissemination

Ethical approval was obtained from the Medical Ethical Committee of the Netherlands Cancer Institute. Independent of the outcome, results of this study will be shared and submitted for publication in an international peer-reviewed journal.

All essential documents (including patient files, the Investigator Study File, CRF's and electronic study data), data management and statistical files will be kept for 15 years.

Summary

Angiosarcoma is an extremely rare and aggressive malignancy with a high metastatic potential and a dismal prognosis. The current standard treatment cannot sufficiently manage the disease. Therefore, new strategies are warranted. Drug repurposing is a process of developing approved drugs for new medical indications. A strong rationale for repurposing of propranolol for the treatment of angiosarcoma patients exists. The precise effect of propranolol monotherapy is not yet established. In this study, we will therefore address the question about the efficacy of propranolol as neoadjuvant monotherapy in patients with cutaneous angiosarcoma. If this study shows positive results, further clinical trials are needed to establish the role of propranolol in the treatment of angiosarcoma, possibly even in combination with other agents such as chemotherapy, targeted therapy or immunotherapy.

Acknowledgments

The authors would like to thank the Dutch sarcoma patient advocacy group (Patiëntenplatform Sarcomen) and the Anticancer Fund for their contributions to the study.

Funding statement

This work was supported by Anticancer Fund from Belgium, grant number 40117.

Competing interest statements

The authors declare that they have no conflicts of interest related to this study.

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Chapter 7
Upregulation of PARP-1 and ALDH1A1 in metastatic sarcoma.

CHAPTER 7

Upregulation of PARP-1 and ALDH1A1 in metastasis of sarcoma patients: new targets for study treatment.

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Research in clinical proteomics.
In preparation.

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Abstract

Background: Soft tissue sarcoma is a group of mesenchymal malignancies consisting of over 70 different subtypes. Each of these subtypes has distinct biologic characteristics and behavior, leading to varying sensitivity to systemic therapy. Treatment of metastatic sarcoma is challenging, partly due to this high variety in subtypes. This study is designed to find common upregulated pathways in the metastases of different sarcoma subtypes, in order to unravel sarcoma metastases biology and identify promising treatment targets for metastatic sarcoma.

Methods: Frozen tissue from surgical specimens or biopsies of patients with untreated primary sarcoma and matching metastases were collected. Proteins were extracted from these tumor samples. Proteomics based on one-dimensional gel electrophoresis coupled to nano liquid chromatography tandem mass spectrometry was used to identify proteome differences between the primary tumors and their metastases. Subsequent data and pathway analysis was performed with David bioinformatics resources and STRING. Promising upregulated targets were confirmed with immunohistochemistry and tested in cell lines with a viability assay.

Central findings: Out of a total data set of 7772 identified proteins, 309 proteins were at least significantly 1,5 fold up-regulated in the metastases, when compared with their primary tumors. Analysis for activated cellular networks in metastases revealed a significantly higher expression of proteins involved in metabolism and cellular respiration, chromatin remodeling, chromosome organization and DNA repair, like PARP-1 and ALDH1A1. Interestingly, PARP-1, an enzyme that plays a role in the detection of DNA damage and repair, was highly upregulated in all tumors except for synovial sarcoma.

Conclusions: Sarcoma metastases show higher expression of proteins involved in DNA repair and chromatin remodeling, suggestion higher chromosome instability in metastases when compared to primary tumors. Treatment with well-known inhibitors targeting PARP-1, might be beneficial for patients with metastatic sarcoma.

Abbreviations

ALDH1A1	Aldehyde dehydrogenase 1
CDK 4/6	Cyclin-dependent kinase 4/6
CNV	Copy number variation
FFPE	Formalin-fixed paraffin-embedded
IHC	Immunohistochemistry
MAPK	Mitogen-activated protein kinase
PARP-1	Poly(ADP-ribose) polymerase-1
PDGFR	Platelet Derived Growth Factor Receptor
PDGFRa	Platelet Derived Growth Factor Receptor Alpha
STRING	Search tool for the retrieval of interacting genes/proteins

Introduction

Sarcoma is a group of malignancies with a mesenchymal origin and consists of more than 70 different histologic subtypes. Each of these histologic subtypes has distinctive biologic characteristics and behavior, leading to varying sensitivity to systemic therapy [1–3]. Surgery, with or without radiotherapy, is standard of care for sarcoma patients with localized disease, but more than half of all sarcoma patients develop distant metastases [4,5]. The treatment of metastatic sarcoma is more challenging, partly due to the high variety in histologic subtypes, and consists of cytotoxic chemotherapy, such as doxorubicin, ifosfamide, or targeted therapy, like pazopanib [6,7]. Unfortunately, the response rates of these therapies are low to moderate, 10-50% [8]. Therefore, there is an urgent need for new treatment strategies to improve the outcome of sarcoma patients.

Fortunately, there are some success stories in the last couple of years. New therapies arise directed against sarcoma specific mutations, based on increased understanding of tumor biology. The introduction of imatinib for example, directed against the KIT mutation, has dramatically changed the survival of gastro-intestinal stromal tumor patients by triplicating the median survival [9]. The CDK 4/6-inhibitor palbociclib shows promising results in several phase II clinical trials for differentiated and dedifferentiated liposarcoma patients, with improving the 12-weeks progression free survival to 57.2-66% [10,11].

However, the search for new targets to improve the treatment of sarcoma patients still continues. This study was designed to identify promising targets for the treatment of metastatic sarcoma. Therefore, we compared protein and gene expression patterns of primary sarcomas with the patterns in the metastases from the same patients. The aim of this study was to determine common upregulated pathways

in the metastases of sarcoma patients, in order to unravel the biology of sarcoma metastases and to identify new treatment strategies.

Experimental procedures

Specimen collection

Fresh frozen tumor tissue was collected from primary and metastatic lesions of sarcoma patients to develop a subset of matched (primary plus metastases) tumor samples of the same patient. Tumor tissue was collected during surgery or by biopsy of an untreated tumor lesion. All histologic subtypes were eligible. These matched tumor samples were used for the proteomics, sequence and IHC experiments. This study was conducted in accordance with the International Conference of Harmonization of Good Clinical Practice and the Declaration of Helsinki.

Identification of common upregulated pathways

We collected fresh frozen tissue from seven primary tumors of sarcoma patients, and of nine metastases of the same patients. These sixteen tumor samples were used for the proteomics and sequence experiments to identify common upregulated pathways.

Proteomics

We used an one-dimensional gel electrophoresis coupled to nano liquid chromatography tandem mass spectrometry to identify the proteome differences between the primary tumors (seven) and their matched metastases (nine).

Subsequent data and pathway analysis was performed with David bioinformatics resources [12], a functional clustering tool allowing the grouping of functionally associated proteins and/or annotation, and with the STRING [13], which visualizes the protein networks/clusters with their specific function and provides a quantitative score to the different interactions.

Copy number variation sequencing

For CNV sequencing, the same sixteen tumor samples were processed into FFPE tumor blocks and DNA was extracted from these tumor blocks. Next generation sequencing was performed on Illumina HiSeq 2000 sequencers and was used to determine copy number variations.

Confirmation of the revealed promising upregulated pathways

We investigated the presence of promising upregulated in sarcoma with immunohistochemistry. Next to the sixteen matched samples, which were used in the proteomics and sequence experiments, an additional set of matched sarcoma tumor

samples was added for the immunohistochemistry assay. The 'old' subset of matched tumor samples was stained to confirm the data obtained with the proteomics and sequence analysis. The addition of the 'new' subset of matched tumor samples was used to further substantiate the importance and the abundance of these upregulated pathways in metastatic sarcoma.

Proof of concept

We analyzed the treatment response to a targeted therapy directed against one of the promising targets, as a proof of concept experiment. For these experiments sarcoma cell lines were used, which were locally available in the Netherlands Cancer Institute. The presence of the revealed upregulated pathway in the sarcoma cell lines was confirmed by western blot analysis. Growth inhibition after exposure to olaparib was measured in a sensitivity assay using cell titer blue staining.

Experimental Design and Statistical Rationale

Sixteen tumor samples of sarcoma patients were used for the proteomics and sequence experiments, fourteen samples for the immunohistochemistry assay and five cell lines for the proof of concept experiment. David informatics and STRING were used for the statistical analysis with internal controls of the proteomics and sequence data. The sensitivity assay was performed in triplo and the results of the western blot were confirmed with tubulin and an additional second antibody. Randomization was not applicable for this study.

Results

Specimen collection

Sixteen tumor samples (seven primary tumors and nine matched metastases) were used in the initial experiments for the identification of common upregulated pathways. For the confirmation experiment with immunohistochemistry an additional five tumor samples were included. An overview of the tumor samples was provided in *Table 1*.

Identification of common upregulated pathways

Proteomics

The separation of primary and metastatic sarcoma by cluster analysis of all significantly up- and downregulated proteins ($p<0.05$) was shown in *Figure 1*. Both supervised and unsupervised clustering resulted in a clear separation between primary and metastatic sarcoma based on protein expression. The quantification of the proteome differences between primary and metastatic sarcoma was shown in *Table 2*.

Table 1. Overview of sarcoma tumor samples. Table 1 gives an overview of the sarcoma tumor samples which were used in the exploration (proteomics and sequencing) and confirmation (IHC) experiments to identify common upregulated pathways.

Histologic subtype	Experiment
Leiomyosarcoma 1	Confirmation
Leiomyosarcoma 2	Confirmation
Malignant peripheral nerve sheath tumor 1	Confirmation
Malignant peripheral nerve sheath tumor 2	Confirmation
Malignant peripheral nerve sheath tumor 3	Confirmation
Myxofibrosarcoma 1	Exploration
Myxofibrosarcoma 2	Confirmation
Myxoid liposarcoma 1	Confirmation
Sarcoma not otherwise specified 1	Exploration and confirmation
Sarcoma not otherwise specified 2	Exploration and confirmation
Solitary fibrous tumor 1	Exploration and confirmation
Solitary fibrous tumor 2	Confirmation
Synovial sarcoma 1	Exploration
Synovial sarcoma 2	Confirmation
Undifferentiated pleomorphic sarcoma 1	Exploration and confirmation
Undifferentiated pleomorphic sarcoma 2	Exploration and confirmation

Subsequently, the significantly up- and downregulated proteins and the networks they relate to, were mapped into a string map, using the data and pathway analysis tools David bioinformatics and STRING (*Figure 2*). Proteins which were significantly higher expressed in sarcoma metastases, when compared to their matched primary tumors, were mostly involved in cell metabolism, respiration, PDGF/MAPK signaling, chromatin remodeling and DNA repair (*Figure 3*).

The ten most abundantly expressed and significantly upregulated proteins in the tumor samples of metastatic sarcoma are shown in *Table 3*. Interestingly, PARP-1 was the most abundantly expressed protein in our subset of metastatic sarcoma samples. PARP-1 is an essential protein in DNA repair, suggesting that there is more DNA instability in metastatic sarcoma [14]. This was further substantiated, when the individual levels of PARP-1 in the sixteen sarcoma samples was measured. PARP-1 was

upregulated in the metastases of all tumor types, except for synovial sarcoma (N=2) (*Figure 4*).

The second most abundantly expressed protein was ALDH1A1, which is associated with the stemness in cancer and is important for therapy resistance, cell differentiation and proliferation [15]. In the further experiments, we concentrated on the presence of DNA instability and the abundance of these two proteins in our metastatic sarcoma samples.

Table 2. Proteome differences between metastatic sarcoma and their matching primary sarcoma samples. The numbers refer to all proteins that are 1, 1,5, 2 or 10 fold up (+)- or down(-)regulated in the metastatic lesions when compared to the primary lesions.

Fold change	+/- 1	+/- 1.5	+/- 2	+/- 10
All proteins	7772	4015	2680	1131
p <0.05	470	414	288	120
All upregulated proteins	4995	2652	1715	772
p <0.05	358	309	193	73
All downregulated proteins	2777	1363	965	359
p <0.05	112	105	95	47

Figure 1. Separation of primary and metastatic sarcoma by cluster analysis. A. Heat map of unsupervised cluster analysis of all proteins B. Heat map of supervised cluster analysis of all significantly up- and down regulated proteins (p<0.05). MFS=myxoid fibrosarcoma, NOS=not otherwise specified, SFT=solitary Fibrous Tumor, UPS=undifferentiated pleomorphic sarcoma.

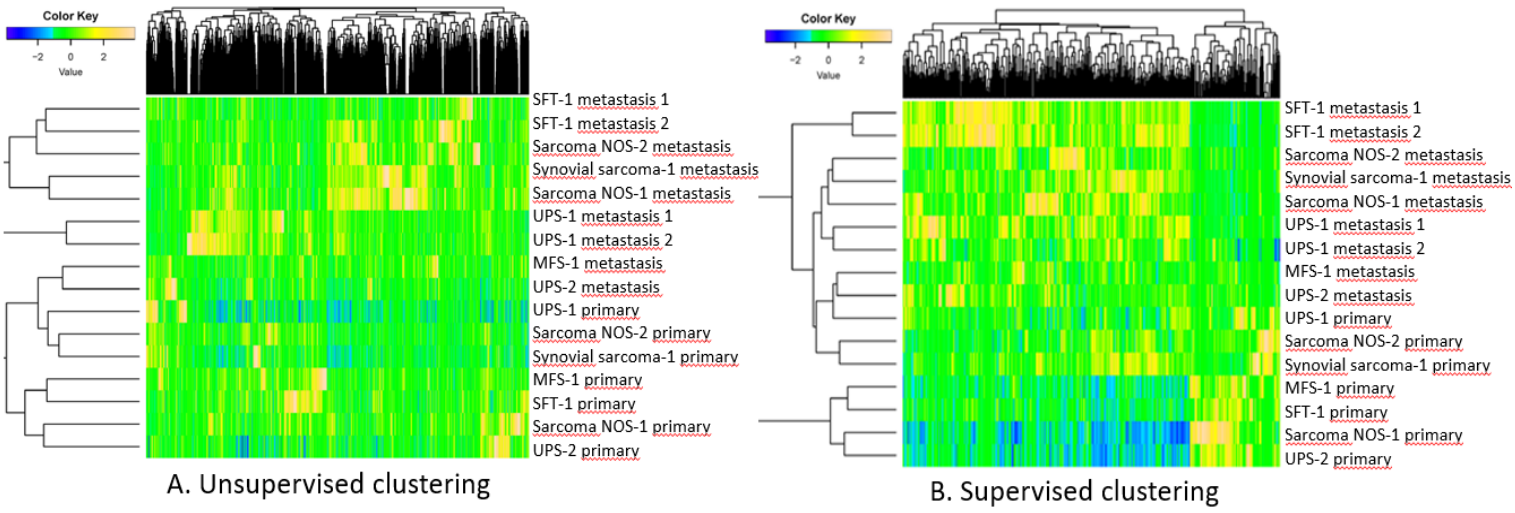
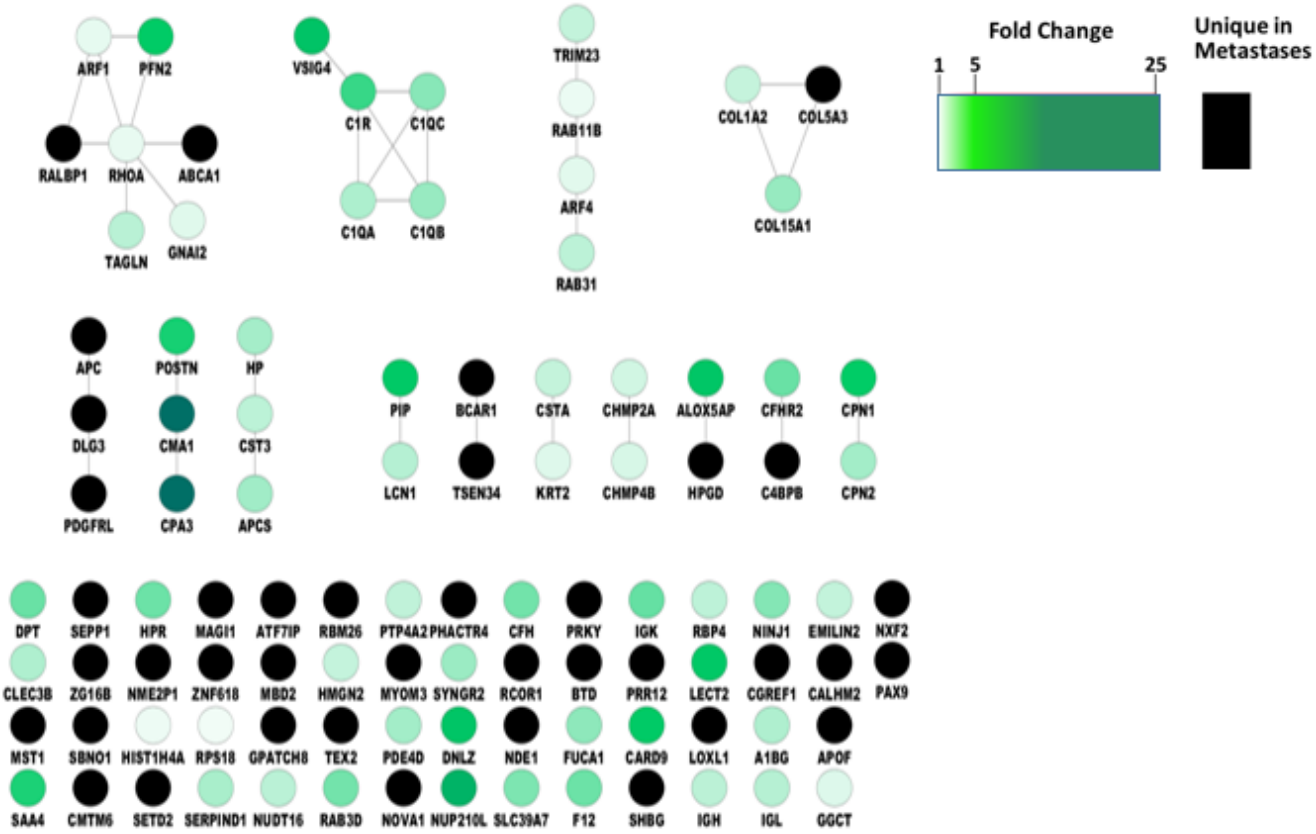


Figure 2. String maps of all significantly up- and downregulated proteins. All significantly up- and downregulated proteins (p<0.05, fold change >1,5 or <-1,5) and the networks they relate to. The upregulated pathways are shown in red. The downregulated pathways are shown in green. The proteins shown in black are unique in the metastatic samples.



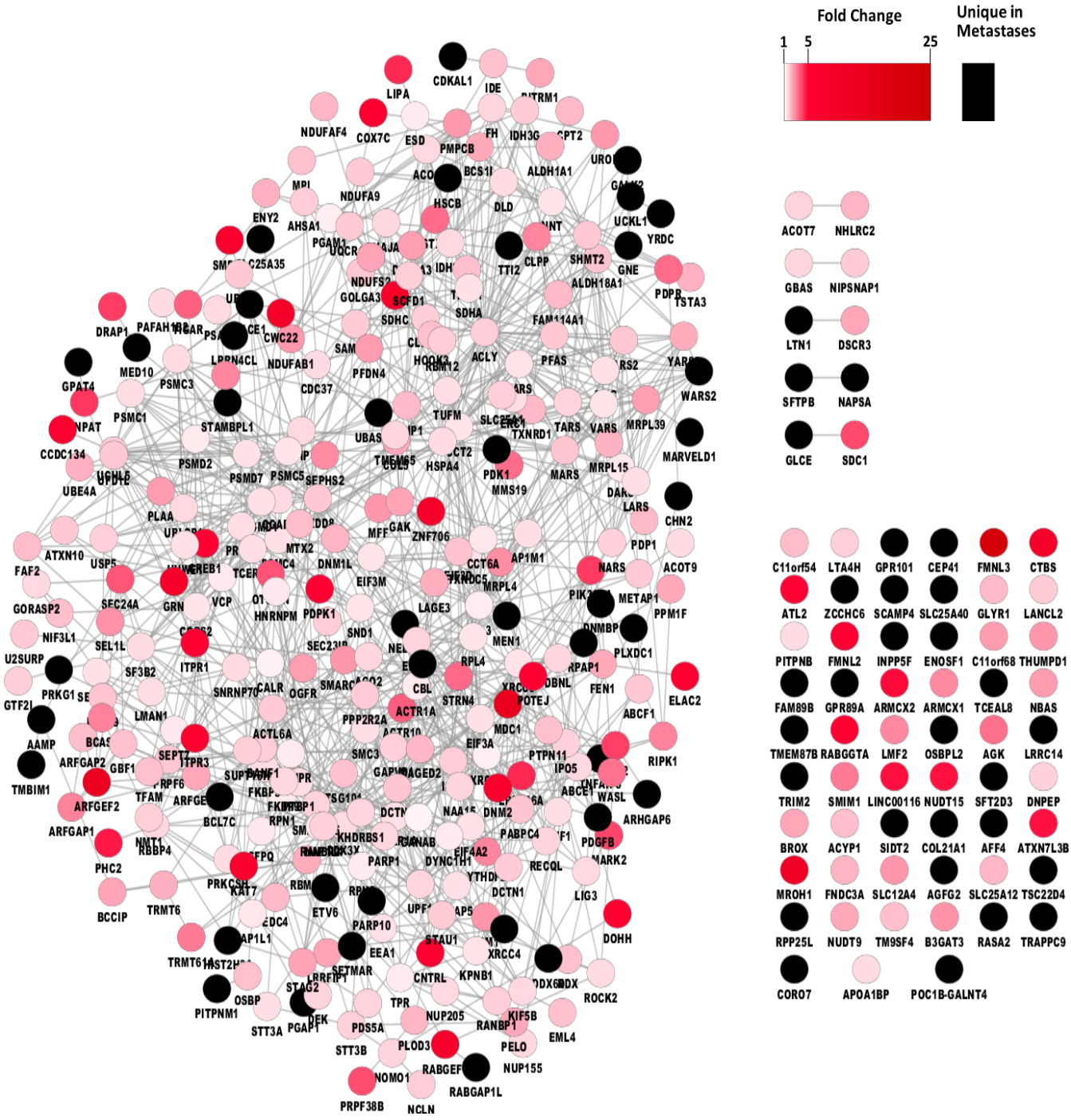
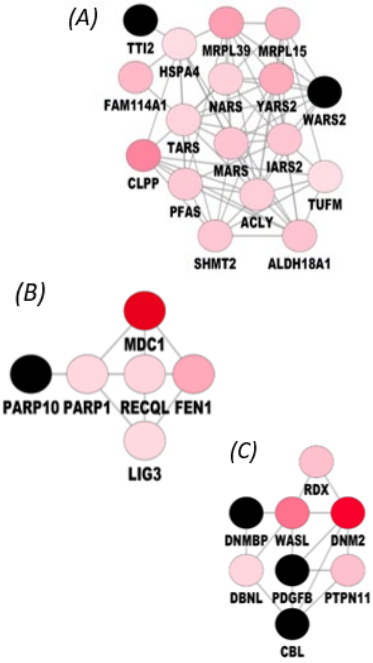

Fold Change
1
5
25
Unique in Metastases
ACOT7
NHLRC2
GBAS
NIPSNAP1
LTN1
DSCR3
SFTPB
NAPSA
GLCE
SDC1
C11orf54
LTA4H
GPR101
CEP41
FMNL3
CTBS
ATL2
ZCCHC6
SCAMP4
SLC25A40
GLYR1
LANCL2
PITPNB
FMNL2
INPP5F
ENOSF1
C11orf68
THUMPD1
FAM89B
GPR89A
ARMCX2
ARMCX1
TCEAL8
NBAS
TMEM87B
RABGGTA
LMF2
OSBPL2
AGK
LRRC14
TRIM2
SMIM1
LINC00116
NUDT15
SFT2D3
DNPEP
BROX
ACYP1
SIDT2
COL21A1
AFF4
ATXN7L3B
MROH1
FNDC3A
SLC12A4
AGFG2
SLC25A12
TSC22D4
RPP25L
NUDT9
TM9SF4
B3GAT3
RASA2
TRAPPC9
CORO7
APOA1BP
POC1B-GALNT4

Figure 3. Functional interactions of up- and downregulated proteins in metastases. Analysis of the functional interactions of up- and downregulated proteins in the samples of the metastases, suggested that there was an up- and down regulation of cellular processes and pathways in metastatic sarcoma.



Upregulated pathways/systems	Downregulated pathways/systems
Metabolism (A)	Rho-signaling pathway (D)
Protein metabolism	Complement activation (E)
DNA repair (D)	Collagen organization
Chromosome organization/remodeling	Interleukin-1 production
Cellular respiration	Microtubule polymerization
PDGF signaling/ activating MAPK pathway (C)	

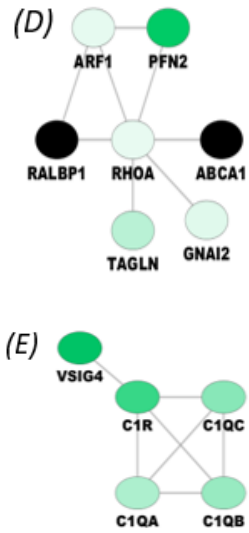
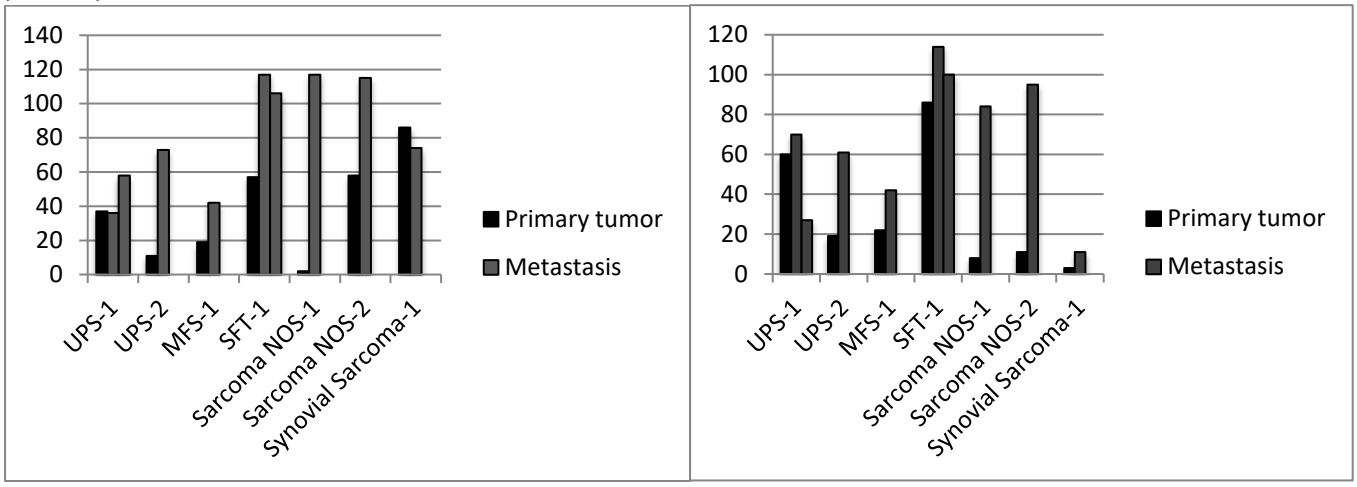


Table 3. Top 10 of upregulated proteins in metastatic sarcoma. Table 3 showed the top 10 of the most abundantly expressed and significantly upregulated proteins in metastatic sarcoma.

Gene name	Protein name	SUM primary	SUM metastasis	Fold change	p-value
PARP-1	Poly [ADP-ribose] polymerase 1	326	681	1.6	0.022995144
ALDH1A1	Retinal dehydrogenase 1	209	603	2.2	0.020364077
PTBP1	Polypyrimidine tract-binding protein 1	273	541	1.5	0.015115593
TUFM	E Elongation factor Tu, mitochondrial	253	494	1.5	0.017474324
EIF3A	Eukaryotic translation initiation factor 3 subunit A	255	488	1.5	0.034567395
ACLY	ATP-citrate synthase	205	462	1.8	0.039615943
ACO2	Aconitate hydratase, mitochondrial	220	456	1.6	0.010702643
PRPF8	Pre-mRNA-processing-splicing factor	222	437	1.5	0.023971359
STIP1	Stress-induced-phosphoprotein 1	205	426	1.6	0.013831837
KIF5B	Kinesin-1 heavy chain	207	424	1.6	0.041134757

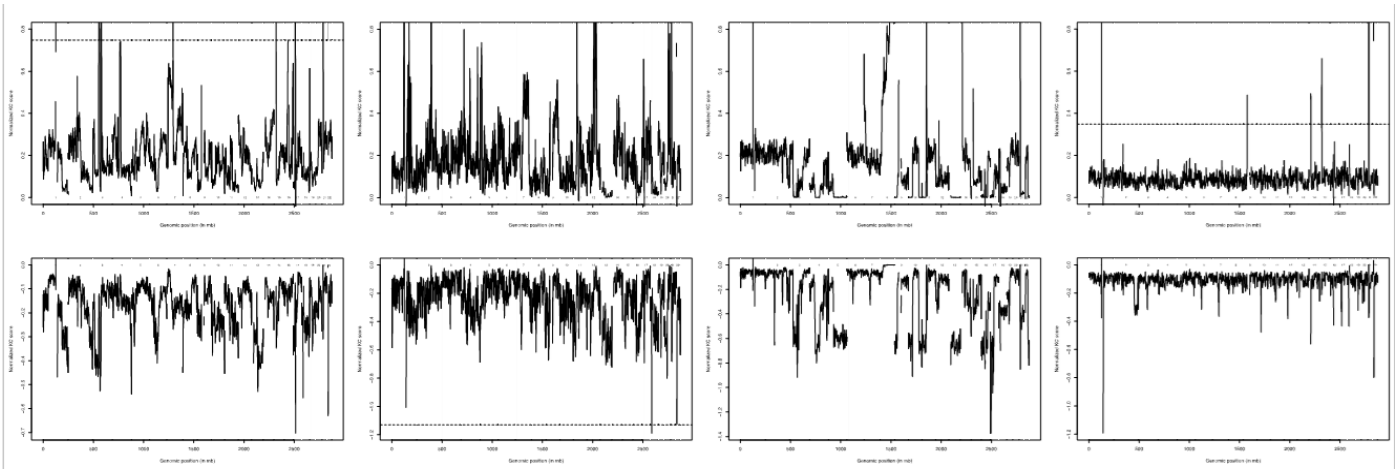
Figure 4. PARP-1 and ALDH1A1 expression in sarcoma. PARP-1 and ALDH1A1 expression in the primary and metastatic sarcoma samples was measured for all tumor types. PARP-1 was upregulated in the metastases of all tumor types except synovial sarcoma-1. ALDH1A1 was also upregulated in metastatic sarcoma, except for one tumor sample of undifferentiated pleomorphic sarcoma-1. MFS=myxoid fibrosarcoma, NOS=not otherwise specified, SFT=solitary fibrous tumor, UPS=undifferentiated pleomorphic sarcoma.



Copy number variation sequencing

Four of the seven sarcoma subtypes (undifferentiated pleomorphic sarcoma, sarcoma not otherwise specified, myxofibrosarcoma and synovial sarcoma) were sequenced to detect DNA instability in the metastases. The chromosomal losses and gains in the samples of metastatic sarcoma were compared with the corresponding primary tumor samples. All the sarcoma subtypes did show increased CNVs in the metastatic sarcoma, except for synovial sarcoma, where there is hardly any DNA instability (*Figure 5*).

Figure 5. Copy Number Variation sequencing plot. Figure 5 shows the chromosomal gains (upper graphs) and losses (lower graphs in metastatic sarcoma compared to the primary tumors. On the Y-axis the normalized KC score, and on the X-axis the genomic position in Mb From left to right: undifferentiated pleomorphic sarcoma 2, myxoid fibrosarcoma-1, sarcoma not otherwise specified 1 and synovial sarcoma 1. All sarcoma types show increased copy number variations in the metastatic tumors, except for synovial sarcoma where there is hardly any DNA instability.



Confirmation of the revealed upregulated pathways: PARP-1 and ALDH1A1

PARP-1 expression was analyzed in fourteen matched tumor samples of sarcoma patients (*Table 4*). In eleven of these patients both the primary and the metastatic lesions were positive for PARP-1. In one patient the primary tumor was negative and the metastatic lesion positive. In two patients the primary lesions were positive but the metastatic lesions turned out to be negative. PARP-1 expression measured with IHC corresponded with the expression found with the proteomics data of the same samples in 67% of the samples.

ALDH1A1 expression was analyzed in thirteen matched tumor samples of sarcoma patients (*Table 4*). In three of these patients both the primary and the metastatic lesions were positive for ALDH1A1. In five patients both the primary and the metastatic lesions were negative. In four patients the primary tumor was negative and the metastatic lesion positive. In one patient the primary lesion was positive but the metastatic lesions turned out to be negative. ALDH1A1 expression measured with IHC corresponded with the expression found with the proteomics data of the same samples in 83% of the samples.

Proof of concept: sensitivity assay for the PARP-1 inhibitor olaparib

As final proof of concept experiment, the sensitivity for olaparib was tested in five cell lines. Two cell lines of metastatic leiomyosarcoma, one of primary undifferentiated pleomorphic sarcoma, one of primary sarcoma not otherwise specified and one of primary myxoid liposarcoma were used.

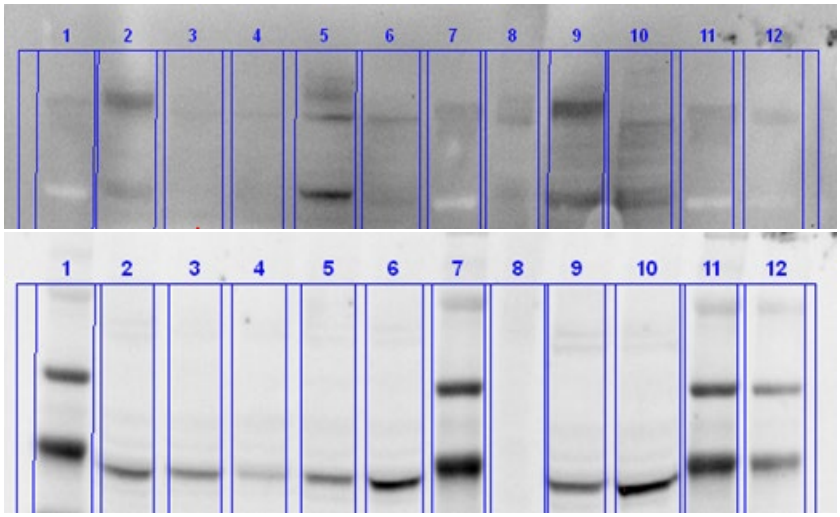
In contrast to our findings in the proteomics and sequence experiments, the metastatic cell lines showed no expression of PARP-1 in the western blot analysis, while the cell lines of the primary sarcoma cell lines were positive for PARP-1 (*Figure 7a*). However, PARP-1 expression in the primary cell lines seemed less surprisingly incorporating the results of the immunohistochemistry assay (*Table 4*), in which most of the primary tumors already show PARP-1 expression.

However, PARP-1 expression seemed to correlate with the responses seen in the viability assay with olaparib (N=5) (*Figure 7b*).

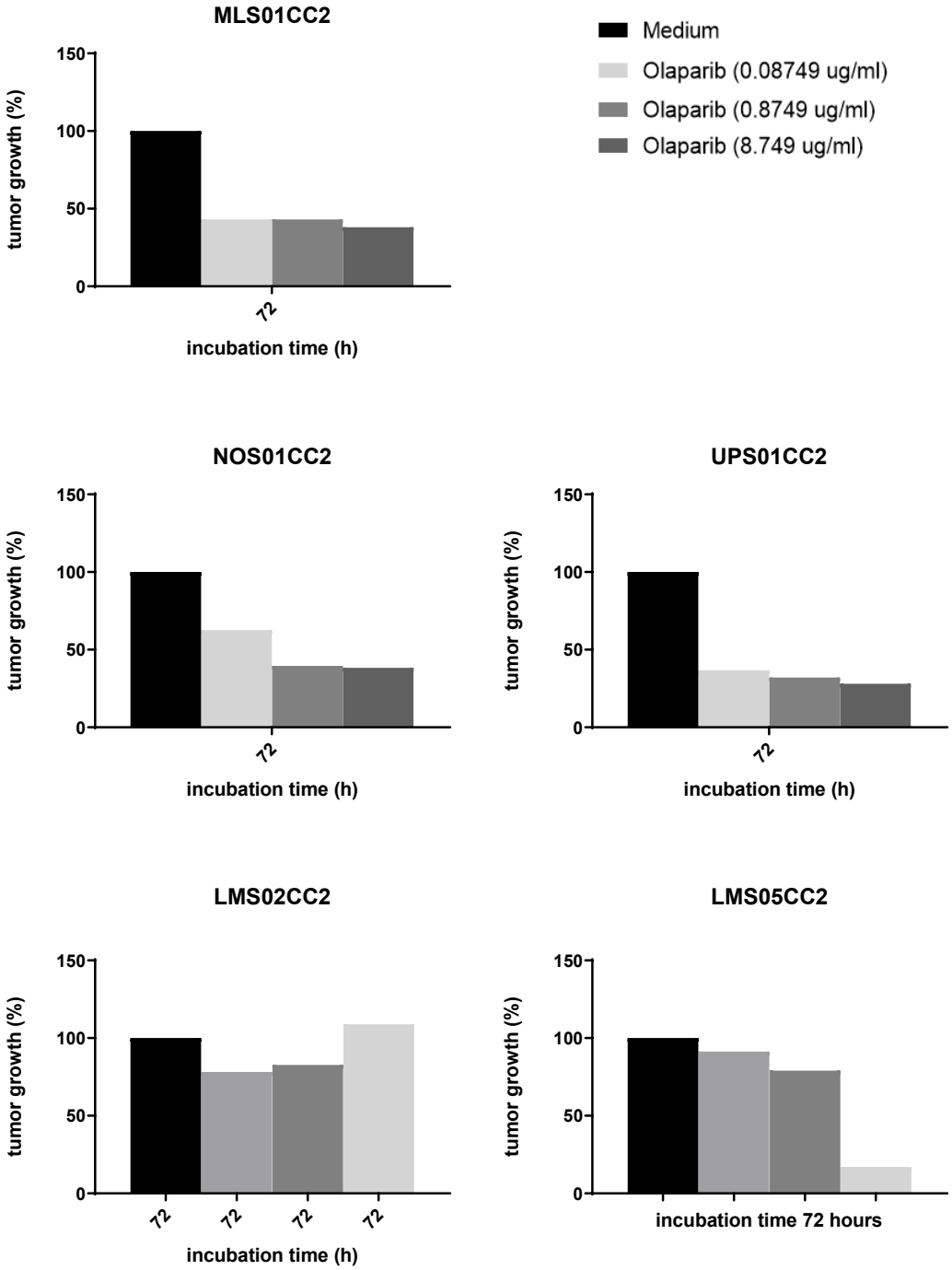
Table 4. PARP-1 and ALDH1A1 expression in sarcoma tumor samples. PARP-1 and ALDH1A1 expression was analyzed in fourteen and thirteen tumor samples, respectively, with IHC assay. * for no correlation between expression measured with IHC and with the proteomics data, ** for a correlation between expression measured with IHC and with the proteomics data. LMS = leiomyosarcoma, MPNST = malignant fibrous tumor, MFS = myxofibrosarcoma, MLS = myxoid liposarcoma, NOS = sarcoma not otherwise specified, SFT = solitary fibrous tumor, SS = synovial sarcoma, UPS = undifferentiated pleomorphic sarcoma.

Histologic subtype	PARP-1 expression in the primary lesion	ALDH1A1 expression in the primary lesion	PARP-1 expression in the metastatic lesion	ALDH1A1 expression in the metastatic lesion
LMS-1	Positive	Negative	Positive	Negative
LMS-2	Positive not specific	Negative	Positive	Negative
MPNST-1	Positive not specific	Negative	Positive	Positive
MPNST-2	Positive	-	Positive	-
MPSNST-3	Positive	Negative	Positive	Negative
MFS-2	Positive *	Positive **	Positive **	Positive **
MLS-1	Positive	Negative	Negative	Positive
NOS-1	Positive *	Negative **	Negative *	Positive **
NOS-2	Positive **	Negative **	Positive **	Negative *
SFT-1	Positive **	Positive **	Positive **	Positive **
SFT-2	Positive	Positive	Positive	Positive
SS-2	Positive	Negative	Positive	Negative
UPS-1	Positive **	Positive **	Positive **	Negative *
UPS-2	Positive *	Negative **	Positive **	Positive **

Figure 7. Sensitivity of sarcoma cell lines for olaparib. 7a. Western blot analysis. The upper figure shows the western blot analysis of PARP-1. The lower figure shows the loading control with tubulin. PARP-1 is visible at 116 kDa (upper bands) and cleaved PARP-1 at 85 kDa (lower bands). Lane 1. lysate, lane 2. UPS01 20ug, lane 3. LMS05 20ug, lane 4. LMS02 20ug, lane 5. NOS01 20ug, lane 6. MLS01 20ug, lane 7. lysate, lane 8. positive control, lane 9. UPS01 30ug, lane 10. MLS01 30ug, lane 11. Lysate, lane 12. lysate. UPS01, NOS01 and MLS02 were positive for PARP-1 and cleaved PARP. LMS=leiomyosarcoma, MLS=myxoid liposarcoma, NOS=sarcoma not otherwise specified, UPS=undifferentiated pleomorphic sarcoma.



7b. Viability assay. The tumor growth after 72 hours of exposure to ten times the IC50 value, the IC50 value and 1/10 of the IC50 value of olaparib was shown in figure 7b for the five sarcoma cell lines. LMS=leiomyosarcoma, MLS=myxoid liposarcoma, NOS=sarcoma not otherwise specified, UPS=undifferentiated pleomorphic sarcoma.



Discussion.

There is an urgent need for new therapeutic strategies in the treatment of metastatic sarcoma. With this study, we show how proteomics could help in unraveling the tumor biology of such a heterogeneous disease as sarcoma to find new treatment strategies. After a broad search for down- and upregulated proteins we focused on the two proteins with the highest significant expression in sarcoma, PARP-1 and ALDH1A1. Additionally, we confirmed the upregulation of PARP-1 and ALDH1A1 with three different methods (proteomics, sequencing and immunohistochemistry). And finally, we performed a proof of concept experiment in which we showed that PARP-1 expression in sarcoma cell lines did correlate with treatment response (N=5).

With this study we have provided a promising subset of new targets for the treatment of metastatic sarcoma. However, additional research and eventually clinical trials are needed before suggestions could be made for adjustments in the standard of care. Furthermore, we focused mainly on PARP-1 in the additional experiments, as targeted therapies directed against PARP-1 are already available. For example, olaparib, talazoparib, veliparib, niraparib and rucaparib are already used for the treatment of other malignancies like ovarian cancer and breast cancer [16,17]. But the development of new therapies directed against the other upregulated pathways could also be promising approach.

In the proteomics and sequence analysis, PARP-1 expression was low in the primary lesions but became upregulated in the metastases of the same patient. Although, this effect was not replicated with IHC, PARP-1 still seems to be a promising target for therapy, because PARP-1 was highly expressed in both the primary as the metastatic lesion of almost all the sarcoma samples. In the current IHC analysis we only scored the samples as positive or negative for PARP-1, while a further subclassification in negative, weakly positive or positive could potentially result in a better correlation between the results found with proteomics and with IHC.

Furthermore, several studies also described a role for PARP-inhibition in the treatment of sarcoma. One study treated chondrosarcoma cell lines with talazoparib. They saw a synergistic response with the combination therapy of talazoparib plus temozolomide or radiotherapy, but the responses were variable [18]. In another study growth inhibition was seen in chondrosarcoma cell lines which were treated with olaparib, and even durable complete remissions in a mouse model with the combination of olaparib and temozolomide [19]. In this mouse model, PARP-1 inhibition even decreased the metastases rate from 75% to 20% compared to PARP inhibition in untreated mice [19]. Camaro et al. also showed an upregulation of PARP in rhabdomyosarcoma. They saw a synergistic effect of olaparib and radiation therapy in two primary rhabdomyosarcoma cell lines [20]. This radiosensitization effect of

olaparib was also seen in cell lines of other soft tissue sarcomas [21]. Another study combined rucaparib with trabectidin in a large subset of sarcoma cell lines with a beneficial effect on inhibiting proliferation and inducing apoptosis [22].

Despite promising preclinical results of PARP-inhibition in sarcoma cell lines, there are only three clinical trials in which PARP-inhibition was tested in patients with sarcoma, and these studies were all limited to Ewing sarcoma. In one trial, twelve Ewing sarcoma patients were treated with olaparib monotherapy with no significant clinical responses [23]. However, these patients were treated with olaparib monotherapy, while most preclinical studies highlight the synergistic effect of the addition of PARP-inhibition to radiotherapy or systemic compounds. The second trial investigated the combination of talazoparib and temozolomide in ten patients with Ewing sarcoma. Two patients showed prolonged stable disease, but there were no objective responses seen [24]. The results of the third study in fourteen Ewing sarcoma patients treated with talazoparib showed four stable diseases, but no objective responses (NCT01286987).

Currently, there are several clinical trials ongoing, investigating PARP-inhibition with or without the addition of other compounds. One study investigates the effect of olaparib monotherapy in patients with advanced refractory or relapsed solid tumors, including sarcomas (NCT03233204). Two studies administer a PARP-inhibitor in combination with temozolomide in Ewing sarcoma, rhabdomyosarcoma and leiomyosarcoma (NCT01858168, NCT03880019). In another phase I trial irinotecan is added to this regimen (NCT02044120). In the TOMAS trial the combination of olaparib and trabectidin in advanced and metastatic sarcoma is investigated (NCT02398058). And the triple therapy of olaparib, durvalumab and cediranib is tested in patients with leiomyosarcoma (NCT03851614). Almost none of these studies used PARP-1 expression as a predictive biomarker for response, while our study showed that there was a correlation between PARP-1 expression and response in five cell lines. Furthermore, as shown in the proteomics and sequence experiments, PARP-1 expression was higher in metastatic sarcoma than in primary sarcoma. Therefore, metastatic sarcoma could be better target population for treatment with PARP-inhibitors. Additionally, these studies could help in further understanding the tumor biology of sarcoma and in improving the treatment and outcome of metastatic sarcoma.

PARP-1 seems to be a promising target in the treatment of sarcoma, but probably not as monotherapy. The best combination partner for PARP-inhibition, the selection of predictive biomarkers and the adequate selection of patients still needs to be elucidated. In our proof of concept experiment, a correlation was seen between PARP-1 expression and treatment response in sarcoma cell lines. Therefore, PARP-1

could be a promising predictive marker for treatment response to PARP-inhibitors in sarcoma patients.

To conclude, with this study we have provided a dataset of proteins which are significantly higher expressed in sarcoma metastases when compared to their matched primary tumors. These proteins are mostly involved in cell metabolism, respiration, PDGF/MAPK signaling, chromatin remodeling and DNA repair. There was a higher degree of DNA instability with an increase in chromosomal gains and losses in the metastases, explaining the upregulation of DNA repair pathways. PARP-1, a protein involved in DNA repair, is upregulated in most metastatic sarcomas suggesting that PARP inhibitors could be of more value in patients with metastatic sarcoma with DNA instability.

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Chapter 8
Conclusions and perspectives.

Conclusions and perspectives.

The prognosis of cancer patients is changing with the rise of new diagnostics, knowledge and therapies. With the enhanced understanding of tumor biology, the treatment strategy becomes more personalized with an improvement of survival of cancer patients as result. This thesis describes various strategies to further improve the treatment of cancer patients.

Sarcoma diagnostic and predictive tools.

Sarcoma is a rare and heterogeneous group of malignancies with over 70 histologic subtypes and varying treatment sensitivity. An early diagnosis would result into a better prognosis and the rise of liquid biopsies as a new diagnostic tool creates the opportunity for early and low-invasive diagnostics. **Chapter 1** shows that circulating tumor RNA from tumor-educated platelets as liquid biopsy could be used for sarcoma diagnostics. Sarcoma could be discriminated from controls with an accuracy of 87%. However, the applicability can probably be further improved and its precise place in sarcoma diagnostics is not yet fully clarified. The optimal application could be in the follow up for tumor recurrence in high risk sarcoma patients with the ultimate goal to diagnose these recurrences at an earlier stage and to minimize the use of other more invasive diagnostic tools. Future research should focus on the quest to find the most optimal biosources and diagnostic tools and its timing and place in sarcoma diagnostics.

As the response rate to treatment is low in sarcoma patients and the right treatment for every individual patients is difficult, we developed a predictive model for treatment response in **Chapter 2**. The goal would be to use patient-derived cell lines, to give a personalized prediction of potential active therapies within a month. At this moment, we have shown that cell lines can be developed from tumor biopsies of sarcoma patients and that treatment response could be accurately predicted in 64-80% of cases. These results are promising and suggest that this strategy could be used as a predictive tool. Moreover, additional experiments are warranted. The cell lines need to be fully characterized and the predictive potential of these 2D cell lines should be compared with other models like mouse models and organoids. In theory, organoids and mouse models, with their more organized structure and homeostasis, could better reflect the human situation compared to the 2D cell lines. Therefore, this could result in a more accurate response prediction and a fast personalized biomarker for drug and radiation sensitivity in sarcoma patients.

Immunotherapy and new combinations.

Since the introduction of immunotherapy, the landscape of cancer treatment has drastically changed. Despite impressive responses, for example in melanoma patients, not all patients respond to immunotherapy and the development of treatment resistance is still an issue which need to be addressed. The current strategy to enhance the efficacy of immunotherapy is by combining it with other therapies.

One strategy is the combination of immunotherapy with chemotherapy. Even though chemotherapy was always feared for its inhibiting effects on the immune system, it also has stimulating effects which can be exploited to enhance the efficacy of immunotherapy. **Chapter 3** shows an overview of the stimulating and inhibiting properties of several chemotherapeutic compounds on the immune system and evaluates potential combination partners for checkpoint inhibition. Chemotherapy could be used to change the tumor microenvironment into a more immunogenic one (inflamed tumors) by either inhibiting immunosuppressive cells and/or activating effector cells, or by increasing immunogenicity and increasing T-cell infiltration. Although most chemotherapeutic compounds did exert immunostimulatory effects, the timing, dose and sequence of administration of chemotherapy and immune checkpoint inhibitors seems extremely important. Most of the current clinical trials are just combining the two types of therapies in the original regimen, which often resulted in less promising enhancement of checkpoint inhibition. For example, an adjusted metronomic dose would augment the immunotherapeutic effect, while normal doses mostly result in bone marrow suppression. Therefore, future studies should focus first on finding the most optimal regimen with adjusted dosing and timing of the compounds to maximize the immunomodulating effects of chemotherapy.

Another strategy was shown in **Chapter 4** with two examples of the combination of immunotherapy with new antibodies directed against costimulatory receptors of the T-cell, OX40 (**Chapter 4.2**) and glucocorticoid-induced TNFR-related protein (GITR) (**Chapter 4.1**). Although these combinations did show additive to synergistic efficacy in preclinical models, these results were not replicated in the clinic. Therefore, the development of these compounds directed against OX-40 and GITR was stopped. Other trials in which immunotherapy is combined with antibodies directed against other costimulatory or inhibitory receptors are still ongoing.

Optimized dosing strategies.

An interesting phenomenon has occurred with the dosing strategy of monoclonal antibodies. During early development, the dosing strategy of chemotherapy was mimicked and monoclonal antibodies were implemented with body-size-based dosing schedules, despite the fact that body size only has minimal effect on drug distribution

and elimination. Taken together with the often broad therapeutic window of monoclonal antibodies, fixed-dosing seems to be justified and has already been implemented for nivolumab and pembrolizumab. Fixed-dosing of nivolumab and pembrolizumab has rapidly been implemented in most Dutch hospitals (**Chapter 5.1**), but on the contrary, fixed-dosing strategies stay behind for the other monoclonal antibodies. In the Netherlands Cancer Institute, fixed-dosing has already been implemented for all monoclonal antibodies used in oncology. A cost analysis study revealed that the implementation of fixed-dosing in the Netherlands Cancer Institute resulted in substantial savings in health care costs of €0,8 to €3,1 million per year (**Chapter 5.2**). Beside the economic advantage of the implementation of fixed-dosing, increased safety, reduced spillage of expensive compounds and improved preparation efficacy are important points to take into account. Based on these advantages and the pharmacokinetic rationale, fixed-dosing should be implemented for all monoclonal antibodies used in oncology.

Repurposing of drugs.

Another important strategy which is applied to improve cancer treatment is the repurposing of drugs, in which "old" therapies are used for new indications. Instead of initiating new time consuming and expensive clinical trials from scratch, the increased understanding of tumor biology could be used to repurpose therapies for new indications. Furthermore, repurposing of drugs is even more important for rare diseases in which large clinical studies are almost impossible to conduct.

An example was given in **Chapter 6.2**, in which propranolol, an off-patent and well-known compound used in the treatment of hypertension, was repurposed for the treatment of infantile hemangioma. Based on several similarities between infantile hemangioma and angiosarcoma and several small case reports, which substantiate the hypothesis that propranolol could also be repurposed for the treatment of angiosarcoma, a proof of principle study was initiated. If this study will succeed, this offers a cheap, fast and safe option for the treatment of the extremely rare malignancy, angiosarcoma.

Unraveling of tumor biology should be the basis for drug repurposing. As shown in **Chapter 7**, proteomics and sequencing of tumor samples of sarcoma patients could be used to find additional targets for treatment. Several promising targets were discovered, like PARP-1, for which a targeted compound was already available. Furthermore, PARP-1 expression was correlated to treatment response to olaparib in sarcoma cell lines.

To conclude, this thesis shows how several novel strategies help to improve the treatment of cancer patients. Unraveling tumor biology and pharmacokinetic

properties of anticancer compounds are key factors in almost all of the studies described in this thesis.

Appendix

Summary

Nederlandse samenvatting

Overview of publications

Dankwoord

Curriculum vitae

Summary

The improved understanding of tumor biology has led to new diagnostic and therapeutic strategies in the treatment of cancer. The more personalized care resulted in an improved survival of cancer patients. The research in this thesis focusses on different strategies to further improve the treatment of cancer patients.

In **Chapter 1** the applicability of circulating tumor RNA from tumor-educated platelets was investigated as a liquid biopsy and its potential in improving sarcoma diagnostics was determined. We showed that the self-learning algorithm ThromboSeg could discriminate sarcoma from controls with an accuracy of 87%. Our data alsoindicates that tumor-educated platelets RNA-based liquid biopsies may enable for sarcoma diagnostics.

Chapter 2 focused on the usage of cell lines as a predictive tool for treatment response of sarcoma patients, to prevent treatment with ineffective therapies and to avert unnecessary adverse events. We established a biobank of 22 sarcoma cell lines with a success rate of 69%, which can be used for further research. We investigated a new culture method in which we used human derived serum instead of the standard fetal bovine serum. This resulted in an increased growth rate of the cell lines with a median difference of six days. We were able to predict treatment outcome accurately in 67% (n=12) for the normal cultured cell lines and in 71% of the cell lines cultured in human derived serum for systemic compounds (n=7), and in 64% (n=11) and in 80% (n=5), respectively, for radiotherapy.

In **Chapter 3** a potential new treatment strategy was discussed to enhance the efficacy of checkpoint inhibitors in patients with solid tumors, by combining them with chemotherapy. Despite the well known inhibitory effects of chemotherapy on the immune system, there are also some promising stimulatory effects which could be applied to change the tumor microenvironment into a more immunogenic one. In this review we provided an overview of the stimulatory and inhibitory effects of chemotherapy on the immune system and evaluated potential combination partners for checkpoint inhibitors. The majority of chemotherapeutic drugs has been shown to exert immunostimulatory effects, either by inhibiting immunosuppressive cells and/or activating effector cells, or by increasing immunogenicity and increasing T-cell infiltration. However, the timing, dose and sequence of administration of chemotherapy and immune checkpoint inhibitors seems important, but is still poorly understood.

Chapter 4 described two phase I and early phase II clinical trials in patients with solid tumors, in which checkpoint inhibitors are combined with new antibodies directed against costimulatory receptors of the T-cell. In the study described in **Chapter 4.1** nivolumab was combined with a costimulatory antibody directed against glucocorticoid-induced TNFR-related protein (GITR). The objective was to assess the safety, tolerability and activity of anti-

GITR as monotherapy and in combination with nivolumab. In total 292 patients were included. Anti-GITR had a manageable safety profile, with no grade 3 to 5 treatment-related adverse events for the monotherapy. However, the efficacy was comparable to historical data reported for nivolumab monotherapy. In **Chapter 4.2** the new costimulatory antibody directed against OX40 was combined with or without nivolumab, with or without ipilimumab. The purpose of this study was to evaluate the safety and activity of anti-OX40, alone or in combination with nivolumab, with or without ipilimumab in patients with solid tumors. In total, 20 patients received the anti-OX40 monotherapy and 145 patients received combination therapy in various regimens. Both the monotherapy and combination therapy treatment arms showed a manageable safety profile. There were no objective responses seen with monotherapy and the objective responses with combination therapy ranged from 0-13% which was not higher than expected for treatment alone with nivolumab and/or ipilimumab.

In **Chapter 5** a new dosing strategy of monoclonal antibodies used in oncology was discussed. Fixed-dosing instead of body-size-based dosing, a strategy which was originally applied for cytotoxic agents, could be a more optimal strategy for the administration of monoclonal antibodies and is already registered for nivolumab and pembrolizumab. In **Chapter 5.1** the implementation of fixed-dosing was investigated of monoclonal antibodies used in oncology in Dutch hospitals with an online questionnaire. Fixed-dosing of nivolumab and pembrolizumab was rapidly implemented in most Dutch hospitals, but not for other monoclonal antibodies used in oncology. In **Chapter 5.2** the economic impact was further analyzed of the implementation of fixed-dosing for all monoclonal antibodies used in the treatment of cancer in the Netherlands Cancer Institute. Information about the preparations of all monoclonal antibodies since August 2017 was collected from the pharmacy records. The savings in number of vials and correlated costs was calculated with two scenarios, clustering or no clustering of preparations. Fixed-dosing resulted in substantial savings in health care costs, calculated between €0,8 and €3,1 million per year.

Chapter 6 focused on the treatment of the rare malignancy, angiosarcoma. **Chapter 6.1** described a systematic review of literature about the effect of neoadjuvant systemic treatment on the resection margins and survival of angiosarcoma patients. Only seven cohort studies and eighteen case reports could be included. Neoadjuvant chemotherapy could be used for downsizing of the tumor, resulting in improvement of the resection margins. No definitive conclusions could be made about the effect of neoadjuvant systemic therapy on the survival of angiosarcoma patients. **Chapter 6.2** showed the study design of the proof of principle study in which we will evaluate the efficacy of propranolol monotherapy in the treatment of angiosarcoma in the neoadjuvant clinical setting.

In **Chapter 7** we searched for potential new targets in the treatment of metastatic sarcoma. We compared proteomics and sequence data of metastatic tumor samples with samples of the primary tumor of the same patient to find common up- or downregulated pathways. The

significantly upregulated proteins were mostly involved in metabolism and cellular respiration, chromatin remodeling, chromosome organization and DNA repair. Two of the potential and promising targets which we found, were PARP-1 and ALDH1A1. PARP-1 expression was correlated to treatment response to olaparib in sarcoma cell lines.

Finally, conclusions and future perspectives and challenges were discussed in **Chapter 8**.

Nederlandse samenvatting

Door de toegenomen kennis over de biologie van tumoren hebben er grote verbeteringen plaatsgevonden in de behandeling van kankerpatiënten. Dit heeft geleid tot een meer gepersonaliseerde behandeling en daarmee een verbetering in de overleving van kankerpatiënten. Het onderzoek in dit proefschrift richt zich op verschillende strategieën om de behandeling van kankerpatiënten verder te verbeteren.

In **Hoofdstuk 1** is de toepasbaarheid van circulerend tumor RNA, verkregen van 'tumor-educated' bloedplaatjes, als 'liquid biopsy' onderzocht en zijn potentiële rol bepaald in de verbetering van de diagnostiek bij sarcoompatiënten. Het 'self-learning' algoritme ThromboSeq kan sarcoom onderscheiden van controlesamples met 87% nauwkeurigheid. Onze resultaten tonen aan dat circulerend tumor RNA van 'tumor-educated' bloedplaatjes als 'liquid biospy' gebruikt kan worden in de diagnostiek van sarcoompatiënten.

Hoofdstuk 2 beschrijft hoe cellijnen gebruikt kunnen worden om de behandelrespons van sarcoompatiënten te voorspellen, zodat niet-effectieve behandelingen vermeden en daarmee onnodige bijwerkingen voorkomen kunnen worden. We hebben een biobank gecreëerd met 22 sarcoom cellijnen met een succespercentage van 69%. Daarbij is een nieuwe kweekmethode onderzocht, waarin het serum van de patiënt zelf gebruikt is in het medium in plaats van het standaard fetal bovine serum. Dit heeft geleid tot een toename in de groeisnelheid van de cellijnen met een gemiddeld voordeel van zes dagen. De behandelrespons op systemische therapie kon in 67% (n=12) van de cellijnen gekweekt in normaal medium en in 71% (n=7) van de cellijnen gekweekt in het patiënt-eigen serum accuraat voorspeld worden. Voor radiotherapie was dit 64% (n=11) in de cellijnen gekweekt met normaal medium en in 80% (n=5) van de cellijnen gekweekt in patiënt-eigen serum.

In **Hoofdstuk 3** wordt een nieuwe behandelstrategie bediscussieerd, waarbij de effectiviteit van checkpoint inhibitie potentieel kan worden verbeterd door de combinatie met chemotherapie in de behandeling van patiënten met solide tumoren. Ook al staat chemotherapie vooral bekend om zijn inhiberende effecten op het immuunsysteem, toch zijn er ook enkele veelbelovende stimulerende effecten beschreven. Zo zou chemotherapie het micromilieu van de tumor meer immunogeen kunnen maken. In dit review hebben we een overzicht van de beschreven inhiberende en stimulerende effecten van chemotherapie op het immuunsysteem weergegeven en potentiële kandidaten voor combinatiebehandeling met checkpoint inhibitie geëvalueerd. Het merendeel van de chemotherapeutica laat enige immuunstimulerende effecten zien, zoals inhibitie van immuunsuppressieve cellen, activatie van effector cellen, toename van immunogeniciteit en toename van tumor infiltratie door T-cellen. Echter is de timing, volgorde en dosering van de toediening met chemotherapie en checkpoint inhibitie erg belangrijk voor het effect van de combinatiebehandeling en dient dit in vervolgstudies verder onderzocht te worden.

Hoofstuk 4 beschrijft twee fase I en vroeg-fase II studies in patiënten met solide tumoren waarbij checkpoint inhibitie wordt gecombineerd met nieuwere antilichamen, gericht tegen costimulatoire receptoren van de T-cel. In **Hoofdstuk 4.1** wordt nivolumab gecombineerd met een costimulatoir antilichaam gericht tegen de glucocorticoïd-geïnduceerd TNFR-gerelateerd eiwit (GITR). Het doel van de studie is om de veiligheid, de verdraagzaamheid en de effectiviteit van anti-GITR als monotherapie en gecombineerd met nivolumab te bepalen. In totaal zijn er 292 patiënten geïncludeerd. Anti-GITR liet een beheersbaar veiligheidsprofiel zien, met geen graad 3 tot 5 behandel-gerelateerde bijwerkingen in de monotherapie behandeling. Echter werd er geen verbetering in de effectiviteit van de combinatietherapie gezien, vergeleken met historische data over nivolumab monotherapie. In **Hoofdstuk 4.2** wordt een nieuw costimulatoir antilichaam gericht tegen OX-40 gecombineerd met nivolumab, plus/min ipilimumab. In deze studie wordt de veiligheid van activiteit van anti-OX40 als monotherapie of gecombineerd met nivolumab plus/min ipilimumab geëvalueerd. Twintig patiënten kregen anti-OX40 als monotherapie en 145 patiënten kregen de combinatietherapie in verschillende behandelschema's. Zowel in de monotherapie schema's als in de combinatietherapie schema's werd een goed veiligheidsprofiel gezien. Er werden geen objectieve responsen gezien in de patiënten die met anti-OX40 monotherapie zijn behandeld. Voor de combinatietherapie cohorten varieerde dit van 0 tot 13%. Dit is echter vergelijkbaar met de respons die verwacht wordt van behandeling met nivolumab plus/min ipilimumab.

Hoofdstuk 5 gaat over een nieuwe doseerstrategie voor monoklonale antilichamen in de behandeling van kanker. Namelijk een vaste dosering (fixed-dosing) in plaats van de initieel geregistreerde doseerstrategie met een dosering aangepast op het lichaamsoppervlak. Deze 'fixed-dosing' strategie is beter geschikt voor monoklonale antilichamen en is reeds geregistreerd voor nivolumab en pembrolizumab. In **Hoofdstuk 5.1** hebben we eerst met een online vragenlijst uitgezocht in hoeverre 'fixed-dosing' van monoklonale antilichamen is geïmplementeerd in de behandeling van kankerpatiënten in de Nederlandse ziekenhuizen. 'Fixed-dosing' van nivolumab en pembrolizumab is snel na de registratie in de meeste ziekenhuizen in Nederland geïmplementeerd, maar dit bleef achter voor de andere monoklonale antilichamen. In **Hoofdstuk 5.2** hebben we de economische impact van de implementatie van 'fixed-dosing' van alle monoklonale antilichamen in de behandeling van kanker geanalyseerd in het Nederlands Kanker Instituut. Informatie over de bereidingen sinds augustus 2017 is uit de database van de apotheek verzameld. Uit deze gegevens hebben we het aantal bespaarde flacons berekend en de gecorreleerde kosten per jaar aan de hand van twee scenario's: een scenario waarin alle bereidingen worden geclusterd per dag en een scenario waarin er geen clustering plaatsvindt. De implementatie van 'fixed-dosing' van alle monoklonale antilichamen in het Nederlands Kanker Instituut heeft geresulteerd in een besparing tussen de €0,8 en €3,1 miljoen per jaar.

Hoofdstuk 6 gaat over de behandeling van de zeldzame maligniteit, het angiosarcoom. **Hoofdstuk 6.1** beschrijft een systematisch review van de literatuur over het effect van neoadjuvante systemische behandeling op de resectieranden en overleving van angiosarcoompatiënten. Er konden slechts zeven cohortstudies en achttien case reports worden geïncludeerd. Neoadjuvante chemotherapie kan zorgen voor een afname van de tumorgrootte, met als resultaat een verbetering van de resectieranden. Er konden geen harde conclusies worden getrokken op basis van de weinige en uiteenlopende literatuur over het effect van neoadjuvante systemische behandeling op de overleving van angiosarcoompatiënten. **Hoofdstuk 6.2** laat het studiedesign zien van een 'proof of principle' studie waarbij het effect van propranolol monotherapie in de behandeling van angiosarcoompatiënten wordt geëvalueerd in de neoadjuvante setting.

Hoofdstuk 7 beschrijft een studie waarbij we hebben gezocht naar potentiële nieuwe targets in de behandeling van gemetastaseerde sarcoompatiënten. Om overeenkomende pathways te vinden die verhoogd of verlaagd tot expressie komen in metastasen van sarcoompatiënten, hebben we proteomics en sequence data vergeleken van gemetastaseerde tumorsamples met de samples van de primaire tumor van dezelfde patiënt. De meeste eiwitten die significant verhoogd tot expressie kwamen, zijn betrokken bij het metabolisme en aerobe dissimilatie, hermodellering van chromatine, organisatie van chromosomen en het herstel van DNA. Twee van de potentieel veelbelovende targets waren PARP-1 en ALDH1A1. In sarcoomcellijnen was PARP-1 expressie gerelateerd met de respons op behandeling met olaparib.

Tenslotte worden de conclusies en toekomst perspectieven en uitdagingen bediscussieerd in **Hoofdstuk 8.**

List of publications

Articles

Published

Heinhuis KM, In 't Veld SGJG, Dwarshuis G, van den Broek D, Sol N, Best MG, van Coevorden F, Haas RL, Beijnen JH, van Houdt WJ, Würdinger T, Steeghs N. RNA-Sequencing of Tumor-Educated Platelets, a Novel Biomarker for Blood-Based Sarcoma Diagnostics. Cancers. 2020 June;12(6):1372.

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Dankwoord

Allereerst wil ik de patiënten en hun families bedanken voor hun deelname aan de verschillende studies. Zonder deze patiënten was dit boekje er nu niet geweest en zij hebben er voor gezorgd dat ik het gemeenschappelijke doel van al deze studies goed voor ogen bleef houden, namelijk het verbeteren van de patiëntenzorg.

Daarnaast wil ik mijn promotor, Jos Beijnen, en mijn copromotor, Neeltje Steeghs, bedanken voor hun begeleiding gedurende mijn promotietraject, hun altijd kritische input en de kans die zij geboden hebben om een zo divers promotietraject te kunnen doorlopen.
Mijn copromotor, Winan van Houdt, bedankt voor de verdieping binnen het gebied van de sarcomen en voor je begeleiding bij de celkweken in het lab.
Ik wil Frans Opdam, Sofie Wilgenhof en Michiel van der Heijden bedanken voor hun begeleiding bij de klinische farmacologie studies.
Ik wil Jos Jonkers, Ellen Wientjens, Rick Haas, Julia Boshuizen, Hilal Colakoglu, Paul Slangen, Mark de Gooijer, Dick Pluim, René Boosman, Bastiaan de Boer en Daniela D'Empaire Altimari bedanken voor hun input en hulp bij de experimenten met de celkweken.

Thomas Würdinger, Sjors in ’t Veld, Myron Best, Nik Sol en Govert Dwarshuis bedankt voor de fijne samenwerking met de VU en het mooie eindresultaat in de vorm van het manuscript over circulerend tumor RNA bij sarcoom patiënten.

Ik wil Winette van der Graaf, Jeroen Hendrikx, Alwin Huitema, Pia Kvistborg, Marleen Kok, Jan Schellens, Martijn Kerst, Yvonne Schrage, Frits van Coevorden en Anne Miek Koenen bedanken voor hun bijdrage aan de studies en de manuscripten.

Ik wil iedereen van de Clinical Research unit, triallab, trialbureau en pathologie bedanken voor hun harde werk en ondersteuning bij de uitvoering van de klinische studies. Ik wil Bianca, Yvonne, Jenny en Martine specifiek bedanken voor de gezellige momenten en leuke gesprekken in het kinetiekhok.

Daarnaast wil ik mijn directe collega’s op H3 bedanken voor het meedenken op de H3 meetings en vooral voor de gezellige tijd met de leuke labstaps en kerstdiners, en Thea, Hans en Lara voor de gehele organisatie op de afdeling.

En natuurlijk wil ik mijn mede-OIO's, Remy, Sven, Maikel, Lotte, Aurelia, Roos, Mark, Vincent, Linda, Jeroen, Emilie, Nancy, Alejandra, Nikki, David, Hedvig, Hinke, Ignace, Jonathan, Julie, Karen, Lisa, Luka, Maaike, Maarten, Merel, Paniz, Semra, bedanken voor de leuke 4 jaar, met de gezellige weekendjes weg en de soms hoognodige koffie/ijspauzes; Artur voor de interessante leermomenten; Sanne, Jill, Jorn en Eveline voor de gezelligheid en goede gesprekken.

En met name mijn kamergenoten, René, Laura, Marit, Steffie, Willeke en Alaa, voor de vele kameretentjes, tripjes naar de markt en lekkere nieuwe recepten.

Mijn paranimfen, René en Darakshan, bedankt voor al jullie hulp, steun, sparmomentjes, gekkigheid, voorbereidende etentjes, de jurk, kortom de gezellige afleiding.

Tenslotte wil ik mijn ouders, broer en zusje bedanken voor hun steun en vertrouwen in mij. En natuurlijk, pap en mam, heel erg bedankt voor de mooie tekening en design van de kaft van mijn boekje.

Curriculum vitae

Kimberley M. Heinhuis was born February 17th 1990 in Nieuwegein and grew up there in the center of the Netherlands. After completing high school at the Anna van Rijn College in Nieuwegein, she moved to Amsterdam to study medicine. In July 2015 she graduated from the VU University of Amsterdam. After this she started as a resident internal medicine at the Sint Lucas Andreas Hospital in Amsterdam. Kimberley started her PhD in June 2016 under supervision of Prof. Beijnen at the Netherlands Cancer Institute. She performed phase I and early phase II clinical trials with immune-oncology in patients with solid tumors and she developed diagnostic and predictive tools in sarcoma patients. In September 2016 she started the training to become a clinical pharmacologist. In September 2019 she finished this study program.