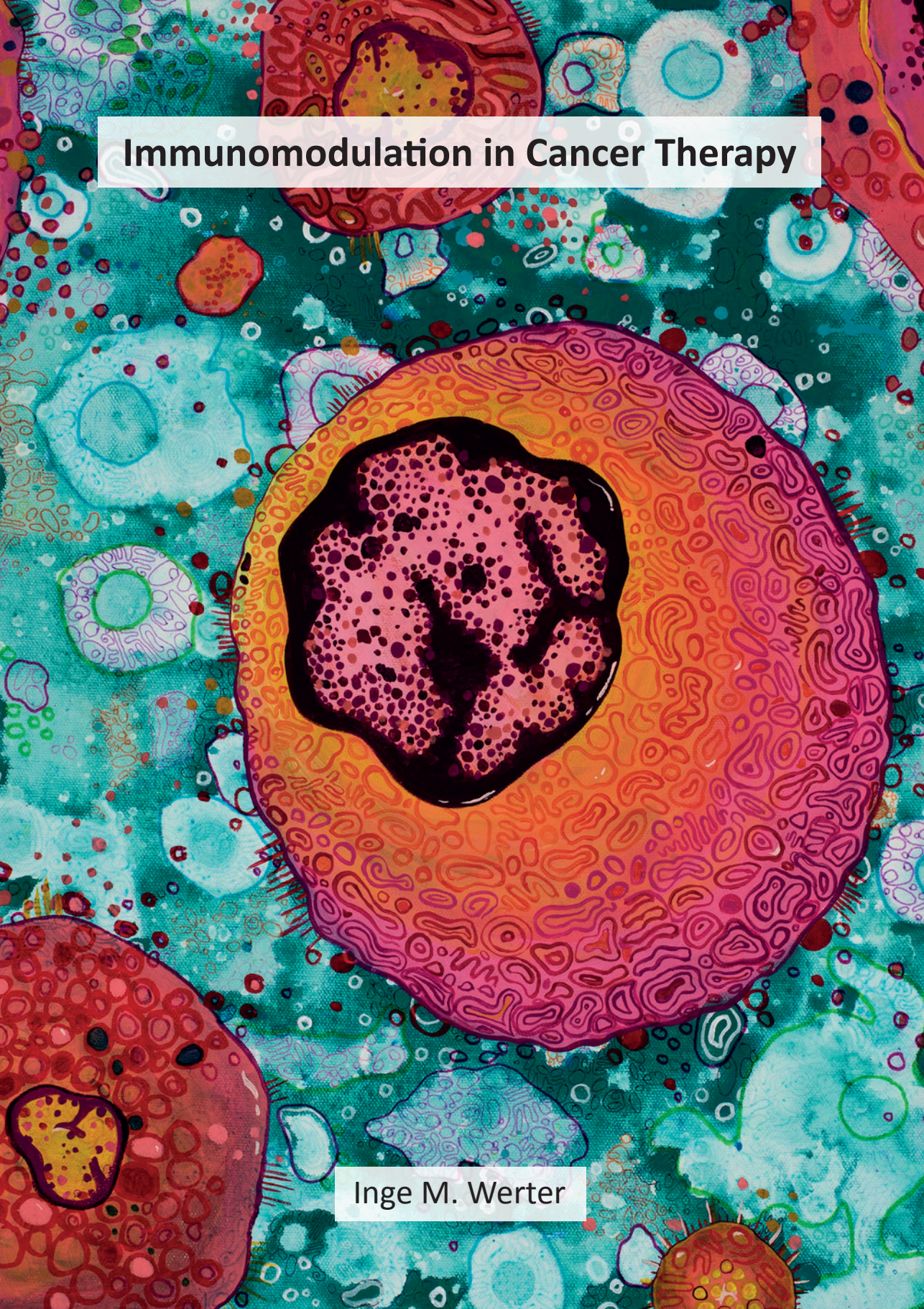
Immunomodulation in Cancer Therapy



Inge M. Werter

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IMMUNOMODULATION IN CANCER THERAPY

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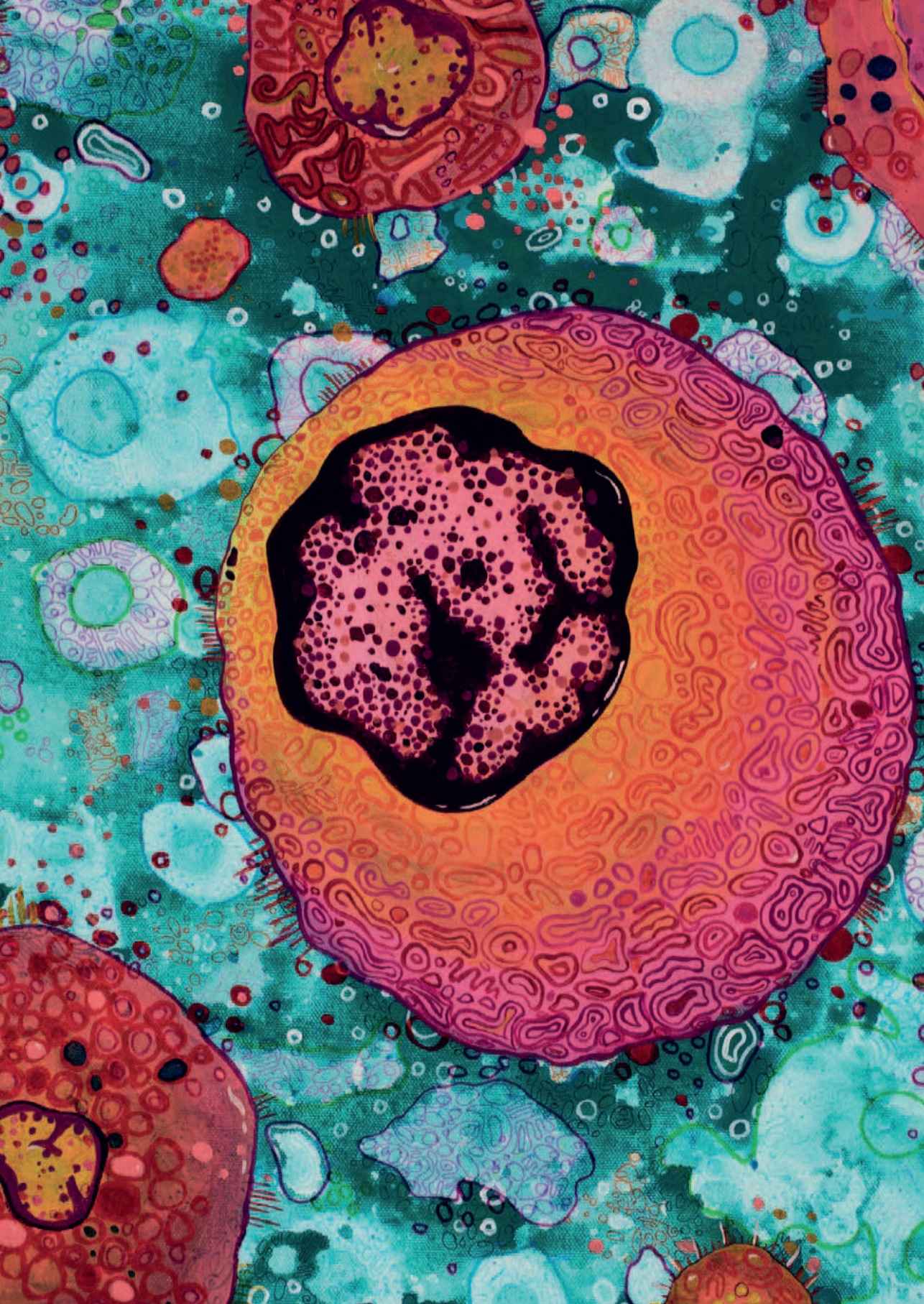

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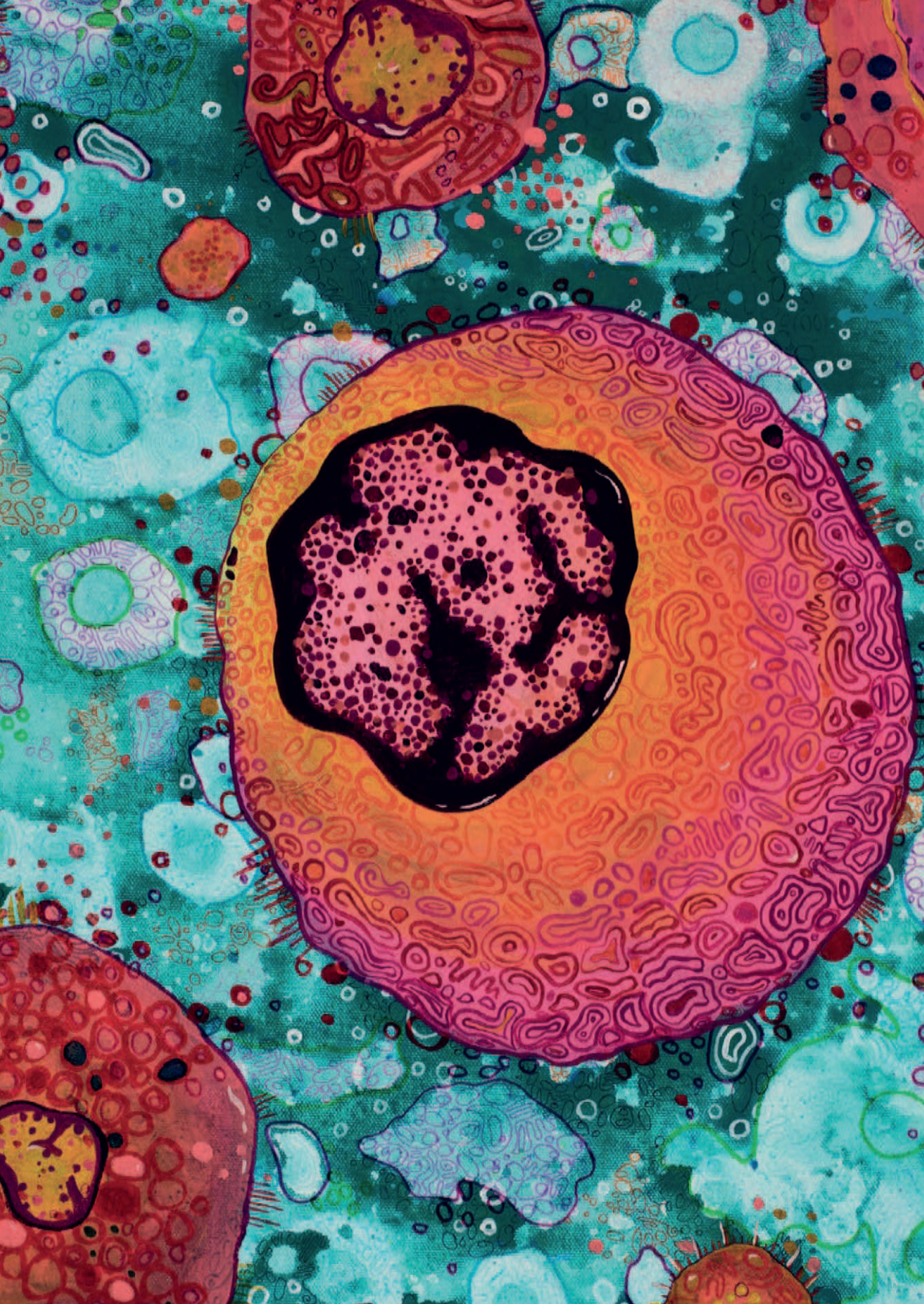
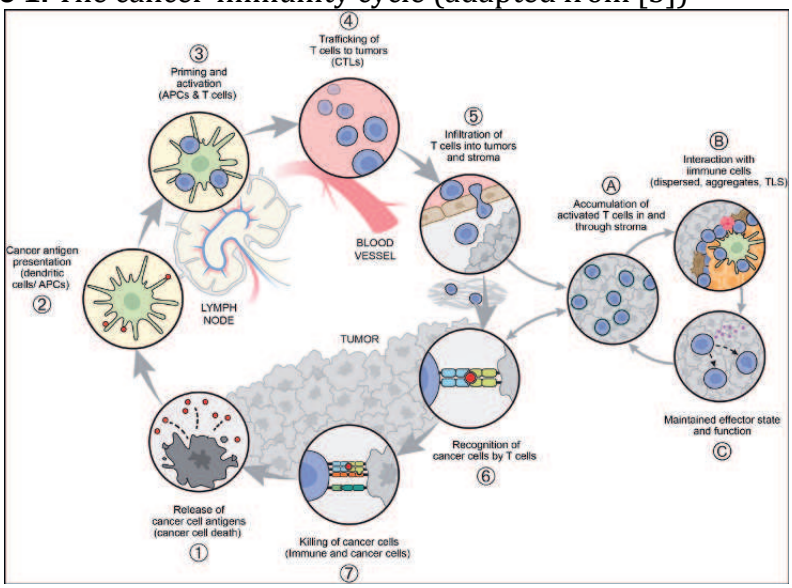
Chapter 1 General introduction and outline of thesis



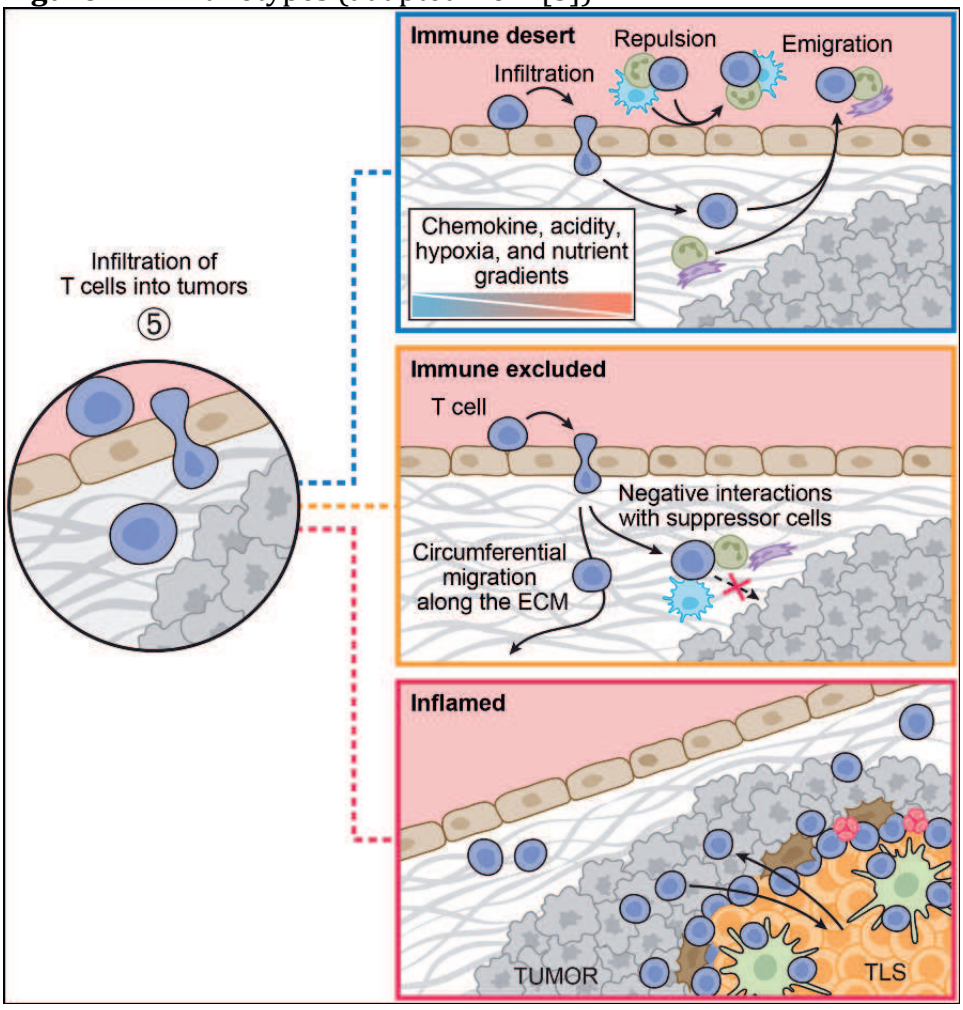
The hallmarks of cancer comprise six biological characteristics acquired during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Another hallmark is the evasion of immune destruction [1]. Cancer can downregulate expression of tumor antigens, produce immunosuppressive cytokines, upregulate expression of inhibitory molecules (immune checkpoints) and promote the expansion and infiltration of suppressor cells in the tumor micro-environment (TME) [2]. Since the breakthrough discovery of the therapeutic efficacy of checkpoint inhibitors (CPI) such as monoclonal antibodies antagonistically binding to programmed death 1 (PD-1), programmed death ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), there has been an enormous surge in studies aimed at boosting the immune response to fight cancer. Since then, the concept of the cancer-immunity cycle (CIC) has been introduced, illustrating that T cells neither respond nor work on their own, but exist in the context of different tissues and undergo a series of steps along their path from priming to the anticancer effector phase, some of which are extrinsic to the immune system and the cancer (Figure 1) [3].

Figure 1. The cancer-immunity cycle (adapted from [3])



As part of understanding the interplay of tumor cells with the immune system within the CIC, classification of tumor immunotypes can be done based on the amount of T cells infiltrating into the TME, namely (1) both stroma and parenchyma (immune inflamed), (2) limited to tumor stroma (immune excluded) or (3) altogether absent immune infiltration (immune desert) (Figure 2) [3]. This variation in immune infiltration contributes to the differential efficacy of CPI across different tumor types, as well as among patients with the same tumor type or between distinct tumors within a single patient.

Figure 2. Immunotypes (adapted from [3])



However, this classification is an oversimplification leading to tumors being referred to as simply being hot (presence of T cells) or cold (absence of T cells). The two tumor types studied in this thesis, are renal cell cancer (RCC) and breast cancer (BC). RCC is considered immune inflamed due to high levels of immune infiltrates and/or the presence of an interferon (IFN)-gamma response signature and therefore can be responsive to CPI [4]. Albeit immunologically inflamed, a significant amount of patients with metastatic RCC (mRCC) cannot be cured with CPI treatment. BC on the other hand is mostly immune excluded or presents with an immune desert immunotype and therefore more difficult to treat with immune modulating strategies like CPI.

In addition to classifying tumor immunotypes based on the quantity of T cells in the TME, the immunosuppressive capabilities of cancer cells and/or the immune system are a contributing factor in the CIC. Tumor cells can create an immunosuppressive environment by producing suppressive metabolites, such as prostaglandin E2, 2-hydroxyglutarate, kynurenine and adenosine or cytokines, like e.g. IL-10, IL-6, VEGF, and TGFβ. Moreover, the TME harbours immunosuppressive non-immune cells such as cancer-associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs) as well as immunosuppressive immune cells such as regulatory T cells (Tregs), regulatory macrophages (Mregs), myeloid derived suppressor cells (MDSCs), tolerogenic dendritic cells (tolDCs), and regulatory B cells (Bregs) [3, 5]. $CD4^{+}CD25^{hi}CD127^{-}FoxP3^{+}$ Tregs represent a functionally distinct lineage of immunoregulatory T cells crucial for maintaining a microenvironment that suppresses the effector function of tumor-infiltrating cytotoxic lymphocytes (CTL) [6]. Elevated frequencies of both circulating and peritumoral Tregs are associated with adverse outcome in several tumor types [6].

Lastly, an important player in modulation of tumor growth and subsequent immune responses in the CIC are the professional antigen-presenting cells, DCs. These cells are key not only for initiating T cell responses early in the CIC (both endogenous and following vaccination) but also for maintaining them, the regulation of DC activation or maturation is considered a key element in driving the CIC [3].

Aims of this thesis

In this thesis different approaches aimed at modulating the immune system in cancer treatment were studied. In the first part of this thesis, we evaluated whether the antitumor efficacy of the mTOR inhibitor everolimus in patients with mRCC could be enhanced by preventing the detrimental everolimus-induced expansion of Tregs through the use of a metronomic cyclophosphamide regimen.

In the second part, we evaluated the effects of the prolonged presence of the primary breast tumor and its draining lymph nodes during neoadjuvant chemotherapy (NAC) and the effect of GM-CSF versus G-CSF administration on the antitumor immune response, and more specifically the functional properties of DCs. Second, the most effective therapeutic strategy in patients with human epidermal growth factor receptor-2 amplified (Her2+) metastatic BC (mBC) with brain metastasis (BM) was evaluated in a systematic review.

Outline of this thesis

Part One: Reversing mTOR inhibitor induced regulatory T cell stimulation using low dose oral cyclophosphamide in patients with metastatic renal cell carcinoma.

The most common tumor arising in the kidney is RCC. RCC is a disease predominantly occurring in men (male:female, 2:1) at a median age of about 60 years. RCC can be classified into four histological subtypes, i.e. clear cell (60-80%), papillary (10-15%), chromophobe (5-10%) and collecting duct carcinoma (< 1%). Approximately 30% of all patients with RCC have metastatic disease at presentation and 30-50% of patients undergoing surgery with curative intent can be expected to experience relapse at distant sites [6]. The treatment of mRCC has changed radically over the past decades. After years of response rates of only 10–20% in mRCC patients treated with interferon-α and interleukin-2, the first series of breakthroughs occurred in 2007 and 2008. The tyrosine kinase inhibitors (TKI) of the vascular endothelial growth factor (VEGF)-signaling pathway, such as sunitinib and pazopanib [7, 8] and inhibitors of the mammalian target of rapamycin (mTOR), such as temsirolimus and everolimus [9, 10] were introduced as first- and second-line treatment options respectively. Since 2018 the landscape has shifted once more, due to the introduction of CPI either alone, in doublet, or combined with TKI [11-14]. At the time when the mRCC studies reported in this thesis were conducted, everolimus was the standard second-line treatment.

Everolimus is an effective inhibitor of mTOR, resulting in the inhibition of proliferation, angiogenesis and survival of tumor cells. In addition, mTOR plays an important role in immune regulation, by balancing effector CTLs and Tregs [15-18]. mTOR inhibition was shown to result in Treg expansion [19-21] and increased levels of Tregs have been associated with diminished survival in cancer patients, including mRCC [22-24]. The unintended Treg expansion induced by everolimus could possibly be counteracted by the addition of metronomic cyclophosphamide. Cyclophosphamide is an alkylating agent of the nitrogen mustard type that is known to selectively deplete Tregs [25]. Although several (pre)clinical studies have shown an inhibitory effect of cyclophosphamide on Tregs, the optimal dosing of cyclophosphamide for this purpose was not known.

In **Chapter 2**, the results of a phase 1 clinical trial in mRCC patients are outlined. This trial was designed to determine the optimal dose and schedule of metronomic cyclophosphamide for selective Treg depletion. We show that, when combined with the standard therapeutic dose of everolimus (i.e. once daily 10 mg orally), the optimal Treg depleting dose and schedule of cyclophosphamide is 50mg once daily. This combination also led to similar rates and severity of adverse events (AE) in comparison with everolimus alone.

The combination of everolimus and cyclophophamide was further evaluated in a phase 2 clinical trial, described in **Chapter 3**. In this trial, mRCC patients were administered 10mg everolimus (once daily, orally) in combination with 50mg cyclophosphamide (once daily, orally). The aim of this trial was to demonstrate that the addition of metronomic cyclophosphamide to everolimus would improve median progression free survival (mPFS) as compared to everolimus monotherapy. In addition, immunomonitoring was performed to evaluate whether immune effects could be related to clinical outcome. Similar to findings from the phase 1 trial, a significant reduction in Treg rates could be demonstrated by the addition of cyclophosphamide, however this did not translate into improved mPFS.

Part Two: Effect of granulocyte (-monocyte) colony stimulating factor and Her2 targeting in patients with breast cancer

BC is the third most common cancer overall; 32% of BC patients present with metastatic disease [26]. Molecular and prognostic classifications have divided BC into three subtypes: (1) Her2 positive (Her2+); (2) hormone receptor positive (HR+) and Her2 negative (HR+/Her2-, also called luminal); and (3) HR and Her2 negative, known as triple negative BC (TNBC) [27]. Over the past two decades, this division has enabled the development of therapies adapted to each subtype. Besides chemotherapy, several targeted therapies are now widely available. For example, Her2-directed antibodies like trastuzumab and pertuzumab for Her2+ BC, and for luminal BC the anti-estrogen tamoxifen, aromatase inhibitors and CDK4/6 inhibitors. Accumulating evidence indicates that the immune system plays a major role in the control of mammary carcinogenesis and tumor progression [28]. While CPI have revolutionized treatment perspectives in e.g. RCC and melanoma, clinical results with CPI in BC patients are still disappointing. Only in the case of TNBC has the PD-1 inhibitor pembrolizumab, when combined with chemotherapy, improved survival in both the metastatic setting and high-risk, early stage BC and is now considered standard of care [29, 30]. There is still an urgent need to further explore the potential of immune modulation in BC.

In **Chapter 4**, results of the phase 3 Spinoza trial are described, in this study the addition of either GM-CSF or G-CSF to NAC was investigated in patient with locally advanced BC (LABC). LABC comprise large tumors, possibly invading nearby tissues or having spread to nearby lymph nodes. With local therapy alone distant metastases usually appear rapidly, indicating that most of these patients already have micrometastases at the time of diagnosis, indicating the need for effective systemic treatment. Although the Spinoza trial was prematurely terminated due to the sudden discontinuation of the production of GM-CSF molgramostim, the immunomonitoring results and long-term survival data still provide relevant insights today. In this trial, GM-CSF administration resulted in more profound loco-regional effects compared to G-CSF, exemplified by higher frequencies of mature $CD1a^+$ conventional DC (cDC) in tumor-draining lymph nodes (TDLN) in GM-CSF treated patients. This may induce a more effective and robust anti-tumor immune response than current G-CSF based NAC strategies, as indicated by a (non-significant) increase in survival for GM-CSF treated patients.

In the Spinoza study, mostly HR+ BC patients were studied, although Her2+ and TNBC patients were also included. HR+ BC is considered less immunogenic than Her2+ or TNBC. For Her2+ BC immunomodulation is not standard-of-care, therefore we sought to find more insight into treatment options in Her2+ BC. Twenty percent of mBC are Her2+, of which 30% develop BM. Patients with BM have a worse median survival and a poorer quality of life compared to patients without BM [31]. In **Chapter 5,** the results of a systematic review and meta-analysis of therapeutic options in Her2+ mBC with BM are summarized. Our analysis revealed trastuzumab-deruxtecan (T-Dxd) to be the most potent drug to induce a response in patients with Her2+ mBC with BM. T-Dxd is an antibody-drug conjugate (ADC) that is composed of a humanized monoclonal antibody specifically targeting Her2, with the same amino acid sequence as trastuzumab, a cleavable tetrapeptide-based linker, and a potent topoisomerase I inhibitor as the cytotoxic drug (payload) [32]. This ADC also enhances antitumor immunity in a mouse model by upregulating MHC class I expression on cancer cells and activation markers on DCs and increasing the prevalence of intra-tumor $CD8^+$ T cells [33].
Whether T-Dxd achieves its effect mostly due to Her2-targeting, or whether its effect on the immune system could also play a role is not known yet. Although BM were always considered challenging for systemic treatment efficacy, due to the blood-brain barrier (BBB), both the ADC T-Dxd as well as the TKI tucatinib demonstrated intracranial efficacy. Despite this progress, survival of patients with BM is still shorter than it is for patients without BM.
Finally, in **Chapter 6** the impact of the findings described in this thesis will be discussed in the context of current and expected future developments in the field of Cancer Immunotherapy.

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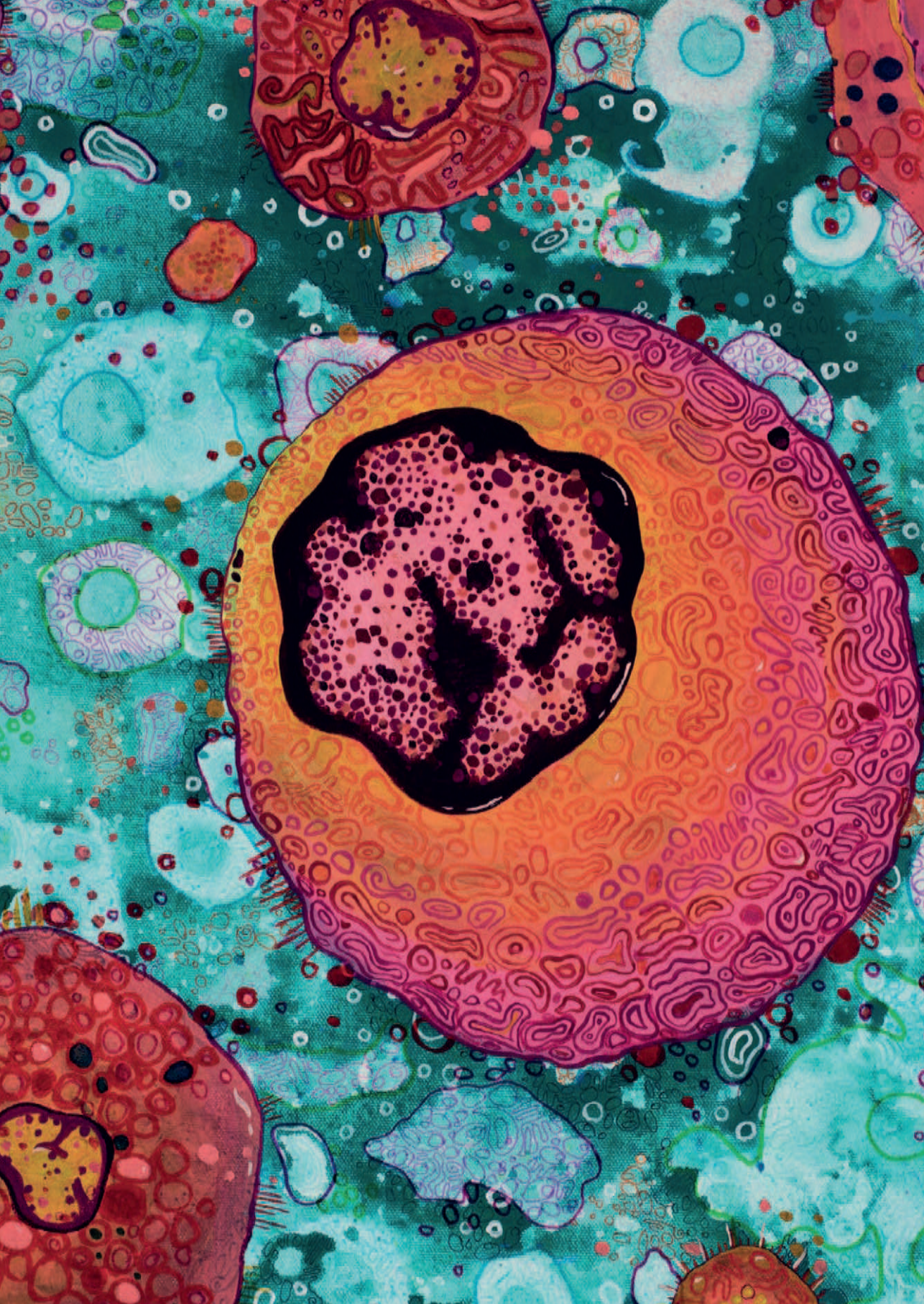
Part One: Reversing mTOR inhibitor induced regulatory T cell stimulation using low dose oral cyclophosphamide in metastatic renal cell carcinoma patients.



Chapter 2 Phase 1 study of everolimus and low-dose oral cyclophosphamide in patients with metastatic renal cell carcinoma

Charlotte M. Huijts, **Inge M. Werter**, Sinéad M. Lougheed, Ruben S. Goedegebuure, Carla M. van Herpen, Paul Hamberg, Metin Tascilar, John B. Haanen, Henk M. Verheul, Tanja D. de Gruijl, Hans J. van der Vliet, for the Dutch WIN-O Consortium
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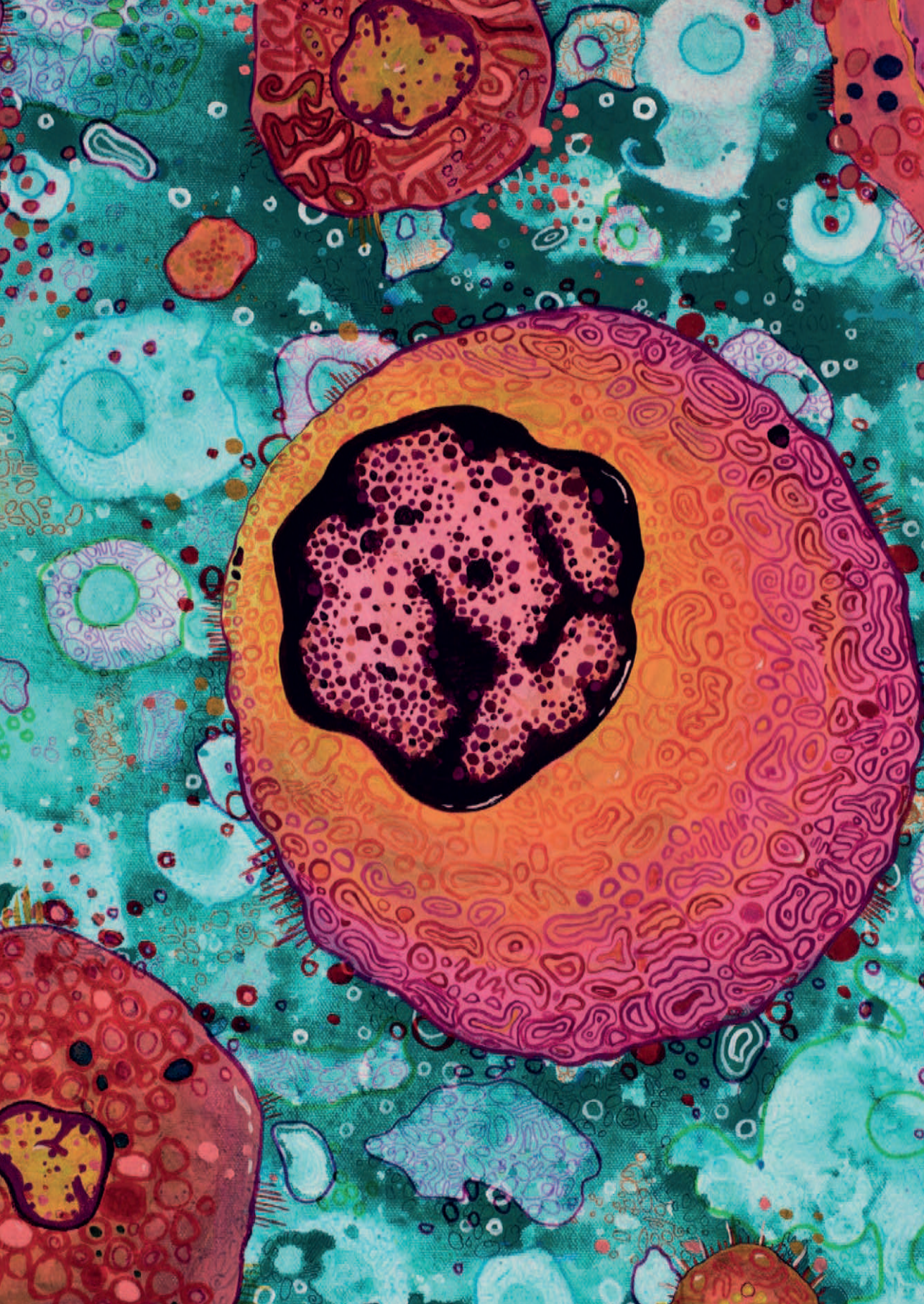

Abstract

Introduction: mTOR inhibitors are frequently used in the treatment of metastatic renal cell cancer (mRCC). mTOR regulates cell growth, proliferation, angiogenesis, and survival, and additionally plays an important role in immune regulation. Since mTOR inhibitors were shown to benefit immunosuppressive regulatory T-cell (Treg) expansion, this might suppress antitumor immune responses. Metronomic cyclophosphamide (CTX) was shown to selectively deplete Tregs. This study was, therefore, designed to determine the optimal dosage and schedule of CTX when combined with everolimus to prevent this potentially detrimental Treg expansion.

Methods: In this national multi-center phase I study, patients with mRCC progressive on first line anti-angiogenic therapy received 10 mg everolimus once daily and were enrolled into cohorts with different CTX dosages and schedules. Besides immune monitoring, adverse events and survival data were monitored.

Results: 40 patients, 39 evaluable, were treated with different doses and schedules of CTX. Combined with 10 mg everolimus once daily, the optimal Treg depleting dose and schedule of CTX was 50 mg CTX once daily. 23 (59%) patients experienced one or more treatment-related ≥ grade 3 toxicity, mostly fatigue, laboratory abnormalities and pneumonitis. The majority of the patients achieved stable disease, two patients a partial response. Median PFS of all cohorts was 3.5 months.

Conclusion: In conclusion, the optimal Treg depleting dose and schedule of CTX, when combined with everolimus, is 50 mg once daily. This combination leads to acceptable adverse events in comparison with everolimus alone. Currently, the here selected combination is being evaluated in a phase II clinical trial.

Introduction

In 2017, 63,990 new cases and 14,400 deaths due to kidney cancer are estimated in the United States and thereby it belongs to the 10 most common cancers in both men and women [1]. The most common tumor arising in the kidney is renal cell carcinoma (RCC). Due to new techniques the histological classification has changed. Though clear cell, papillary and chromophobe RCC are still the most common subtypes, a total of more than 10 subtypes can now be identified [2]. The treatment of metastatic RCC (mRCC) has radically changed over the past 10 years. After years with limited treatment options, when interferon-α and interleukin-2 achieved response rates in only 10–20% of the patients, inhibitors of the vascular endothelial growth factor (VEGF)—signaling pathway and inhibitors of the mammalian target of rapamycin (mTOR), such as temsirolimus and everolimus, were introduced as first and second line treatment options respectively [3]. More recently an inhibitor of the PD-1 immune checkpoint, nivolumab [4], and cabozantinib, a multi-tyrosine kinase inhibitor of MET, AXL and VEGF [5, 6] were shown to be more effective in clinical trials compared to everolimus, thereby replacing everolimus as the standard second line therapy after VEGF targeted therapy [7]. In addition, the combination of everolimus and the multi-target tyrosine kinase inhibitor lenvatinib improved progression-free survival (PFS) in patients with mRCC compared to everolimus alone following one prior anti-angiogenic therapy [8, 9].

Everolimus was shown to be an effective inhibitor of mTOR, resulting in the inhibition of cell growth, proliferation, angiogenesis and survival of tumor cells. In addition, mTOR plays an important role in immune regulation, by balancing effector T cells and regulatory T cells (Tregs) [10–13]. Tregs are important regulators of immunological tolerance and dependent on the transcription factor FoxP3 for their immune suppressive functionality [14, 15]. mTOR inhibition was shown to result in Treg expansion [16–18] and increased levels of Tregs have been associated with poor survival in cancer patients, including mRCC [19–21]. Recently, we and others reported that everolimus leads to Treg proliferation, both in vitro and in vivo [22–24]. Metronomic administration of CTX has been reported to result in Treg depletion, with possible beneficial effects on T- and NK-cell functionality [25, 26].

Therefore, we hypothesized that addition of metronomic CTX to therapy with everolimus in patients with mRCC might counteract the detrimental Treg expansion induced by everolimus and could thereby increase the antitumor efficacy. In this phase I study we aimed to determine the optimal dose of CTX that would result in the selective depletion of Tregs when combined with a fixed dose (10 mg) of everolimus, taking into account the safety and tolerability of the combination treatment.

Patients and methods

Patients

Between January 2012 and August 2015, patients were enrolled in this clinical trial initiated by the department of medical oncology of the VU University Medical Center and conducted within the context of the Netherlands Working Group on Immunotherapy of Oncology (WIN-O) with participation of 13 hospitals. Main inclusion criteria for this trial were an age of 18 years or older, clear-cell mRCC and progression on treatment with a VEGF receptor tyrosine kinase inhibitor. In addition, patients had to have adequate hematologic, hepatic and renal function, measurable or evaluable disease as defined by RECIST 1.1 and a WHO performance status of 0–2. A more detailed description of in- and exclusion criteria can be reviewed in the previously published study protocol [27]. Follow-up was performed until death or at trial analysis, 2 years after inclusion of the last patient.

Treatment

Patients were treated with different doses and schedules of low-dose oral CTX in combination with a fixed dose of everolimus once daily. CTX was either given in a week-on/week-off schedule or continuously and either once or twice daily. These doses and schedules were based on the CTX doses used by Ghiringhelli et al. [26]. Patients were enrolled in cohorts of five patients per dose level. In dose level 6, one patient stopped treatment because of several toxicities (highest grade 3 nausea) within 2 weeks of enrollment and was not evaluable. In case of severe toxicity dose reductions were allowed.

The first five patients were enrolled in an everolimus only cohort with 10 mg everolimus. Subsequently five patients were treated in cohort 1 with the combination of 10 mg everolimus and 50 mg CTX once daily, week-on/week-off. In cohort 2 patients were treated with the combination of everolimus and 50 mg CTX once daily, continuously. In cohort 3 patients received 50 mg CTX twice daily, week-on/week-off, and in cohort 4 patients received 50 mg CTX twice daily, continuously. In the last two cohorts, cohort 5 and 6, respectively, patients received 100 mg CTX twice daily, in cohort 5 in a week-on/week-off regimen and in cohort 6 continuously.

Study objectives

The primary objectives of the study were to determine a recommended dose and schedule for metronomic cyclophosphamide which, when combined with the standard once daily oral dose of 10 mg of everolimus, resulted in optimal and selective Treg depletion in patients with mRCC and to determine the safety and tolerability of this combination. Secondary study objectives included (a) assessment of effects on various immune cell populations, (b) effects on selected angiogenesis parameters, (c) the effect of cyclophosphamide on everolimus drug levels, and (d) clinical outcome measures such as response rate, time to progression, and OS.

Evaluation of toxicity and clinical activity

Patients were treated in cohorts of 5 patients per dose level. In case of no more than 1 dose limiting toxicity (DLT) in a cohort within the 28 days after start of the study treatment, it was allowed to proceed to the next dose level. DLTs were defined as febrile neutropenia, neutropenic infection, other grade ≥ 3 hematological toxicity, pneumonitis, nausea, vomiting, diarrhea, fatigue or any other grade ≥ 3 adverse event that, despite appropriate supportive care, failed to recover to grade ≤ 1 or baseline severity (or grade ≤ 2 at the investigator's and sponsor's discretion) after delaying the next cycle for up to 7 days.

Response to treatment was assessed by the use of RECIST version 1.1. Evaluable patients were defined as those patients completing at least 2 weeks of combination therapy, i.e., allowing the monitoring of immunological effects at time point 2 weeks. Furthermore, patients were evaluated for their performance status, vital signs, general laboratory parameters and immune monitoring at baseline and after 2, 4 and 8 weeks of treatment and every 4 weeks for their clinical condition and general laboratory parameters until the end of study treatment. CT scans of the chest and abdomen were made at baseline and thereafter every 8 weeks. Patients receiving any study treatment were evaluable for safety. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (CTC) grading system version 3.0 (NCI-CTCAE v3.0).

Immune monitoring

Peripheral blood was collected for extensive monitoring at baseline and subsequently at 2, 4, and 8 weeks after the start of the study treatment period and at the end of study treatment. For immune monitoring 60 mL of heparinized peripheral blood was collected. All material was processed on the same day the blood was drawn. In this manuscript, we present immune monitoring data of the effects of the various treatment cohorts on the induction of Treg depletion, the primary objective of this study. The effects of the various treatment cohorts on other immunological parameters will be comprehensively published separately.

PBMC were isolated from heparinized blood of patients by density-gradient centrifugation with Lymphoprep (Axis-Shield, Oslo, Norway). After isolation PBMC were stored overnight at 4 °C in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 100 IU/ml sodium penicillin (Astellas Pharma, Leiden, the Netherlands), 100 mg/ml streptomycin sulfate (Radiumfarma-Fisiofarma, Naples, Italy), 2.0 nM L-glutamine (Life Technologies, Bleiswijk, the Netherlands), 10% FBS (HyClone, Amsterdam, the Netherlands), and 0.05 mM 2-ME (Merck, Darmstadt, Germany), hereafter referred to as complete medium. The next day cells were stained for flow cytometric analysis.

PBMC were analyzed by flow cytometry using FITC-, PerCP- or allophycocyanin (APC)-labeled Abs directed against human CD3, CD4, and CD25 (all BD Biosciences, New Jersey, USA). Stainings were performed in PBS supplemented with 0.1% BSA and 0.02% sodium azide for 30 min. Intracellular staining was performed after fixation and permeabilization using a fixation/permeabilization kit according to the manufacturer's protocol (eBioscience). For staining of FoxP3 a PE-labeled Ab against FoxP3 (clone PCH101, eBioscience) was used. Live cells were gated based on forward and side scatter and analyzed on a BD FACSCalibur (BD Biosciences) using Kaluza Analysis Software (Beckman Coulter).

VEGF measurements

Plasma VEGF concentrations were measured in heparin plasma, frozen the day the material was received and stored at − 20 °C until analysis, using a commercially available ELISA kit (Quantikine, R&D Systems, Abingdon, UK) according to the manufacturers' instructions. Absorbance was measured using a BioTek Synergy HT plate reader with an optical density of 450 nm.

Statistical analysis

One-way repeated measures ANOVA was used to determine the statistical significance of differences within cohorts with Dunnett's Multiple Comparison test as post-test. Two-way ANOVA was used to compare the mean values between cohorts. PFS was defined as time from baseline till progression or death, OS was defined as time from baseline till death. Both PFS and OS were analyzed using Kaplan–Meier curves. Differences were considered statistically significant when p values were ≤ 0.05, as indicated with asterisks (*$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$). Statistical analyses were performed using GraphPad Prism software (version 7, 2016).

Patient characteristics

Between January 2012 and August 2015, a total of 54 patients were screened for this study in 10 different hospitals in the Netherlands. Of these 54 patients, 10 patients did not meet the inclusion criteria while 3 patients withdrew their consent either before start or within the first 2 weeks of study treatment. In addition, 1 patient was excluded because of inadvertent administration of the wrong dose of study medication, while another patient was not evaluable due to early toxicity and subsequent interruption of study medication and withdrawal of informed consent; therefore, 39 patients were analyzed in the study. Patient characteristics are shown in Table 1 and supplementary Table 1. From the 39 patients, 64% were male. The median age of participating patients was 66 years, 20.5% received more than one prior line of systemic therapy, and 72% of patients were in the favorable or intermediate IMDC (International Metastatic Renal-Cell Carcinoma Database Consortium) risk group (Table 1). Patients were discontinued from study therapy because of progression (n = 25, 64%), unacceptable toxicity (n = 12, 30%) or death (n = 2, 5%). Follow-up was performed until death (n = 36) or until time of analysis of the trial (n = 3).

Table 1. Patient Baseline Characteristics

Clinical Characteristics	**Study group (*n* = 39)**
Median age—year (range)	66 (44–78)
Sex—no. (%)	
Male	25 (64)
Female	14 (36)
ECOG performance status—no. (%)	
0	14 (36)
1	20 (51)
2	4 (10)
Unknown	1 (2.6)
IMDC risk group[a]	
Favorable	4 (10)
Intermediate	24 (62)
Poor	9 (23)
Unknown	2 (5)
Median time from initial diagnosis to metastatic disease—months (range)	9 (0–134.5)
Median time from metastatic disease to start of study treatment—months (range)	17 (0.8–290)
Site of metastasis—no. (%)	
Lung	30 (77)
Lymph nodes	24 (62)
Bone	8 (21)
Kidney	7 (18)
Liver	5 (13)
Brain	1 (2,6)
Other[b]	21 (54)
Number of metastatic sites	

Clinical Characteristics	**Study group (*n* = 39)**
1	7 (18)
2	13 (33)
3	9 (23)
≥ 4	10 (26)
Previous systemic cancer therapy	
Sunitinib	33 (85)
Pazopanib	9 (23)
Sorafenib	3 (7.6)
Interferon +/_ bevacizumab	3 (7.6)
IL-2	1 (2.6)
Previous anti-angiogenic regimens—no. (%)	
1	31 (80)
≥ 1	8 (20)

[a]*International mRCC Database Consortium or Heng criteria*
[b]*Adrenal gland, soft tissue, pleural space, muscle, peritoneum/mesenteries, pancreas, vagina, spleen, pericardial tissue*

Treg depletion

The main objective of this phase 1 trial was to determine the optimal dose and administration schedule of orally administered CTX, when combined with 10 mg everolimus, to obtain selective Treg depletion. As shown in Fig. 1a, a (non-significant) increase in Treg percentages within the $CD4^+$ T-cell population was observed in the everolimus only cohort, cohort 0. In cohort 1, 50 mg CTX was administered in a week-on/week-off schedule. Compared to the everolimus only cohort, a significant decrease in Treg percentages at time point 4 was observed. In the next cohort, cohort 2, in which 50 mg CTX was administered in a continuous schedule, a significant decrease in Treg percentages within the cohort was observed when comparing the percentages at time point 0 to time point 4. In addition, a significant difference in Treg percentages between cohort 0 and cohort 2 was observed at time point 4, using the two-way ANOVA. Supplementary Fig. 1. shows representative flow cytometry dot plots illustrating the changes in Treg percentages. Proceeding to the following cohorts, the Treg depleting effect of CTX was progressively less pronounced. Of interest, in the last 2 cohorts, cohort 5 with administration of 100 mg CTX twice daily in a week-on/week-off schedule and cohort 6 with administration of 100 mg CTX twice daily in a continuous schedule, we even observed an increase in Treg percentages. Notably, changes in absolute Treg numbers generally followed the same patterns as observed for changes in Treg percentages. A significant decrease was observed in cohort 2 comparing Treg numbers at week 0 with week 4, while absolute Tregs numbers did not change or even increased in subsequent cohorts (Fig. 2a). Therefore, the decision was made to end the dose escalation phase of the study, and to proceed to the expansion cohort, in which an additional 5 patients were treated with the optimal Treg depleting dose observed in cohort 2. In none of the tested cohorts significant changes in $CD4^+$ T cell percentages were observed. Comparing the $CD4^+$ T cell percentages in the individual cohorts with cohort 0, we did find a significant difference at week 4 between cohort 0 and the expansion cohort (see Supplementary Fig. 2). Lymphocyte percentages increased within cohort 3, 4 and 5 at week 2 and decreased in cohort 6 at week 4. This resulted in significant differences between cohort 0 and cohort 5 and 6 in the first 4 weeks and only at week 4 of the study, respectively (see Supplementary Fig. 3).

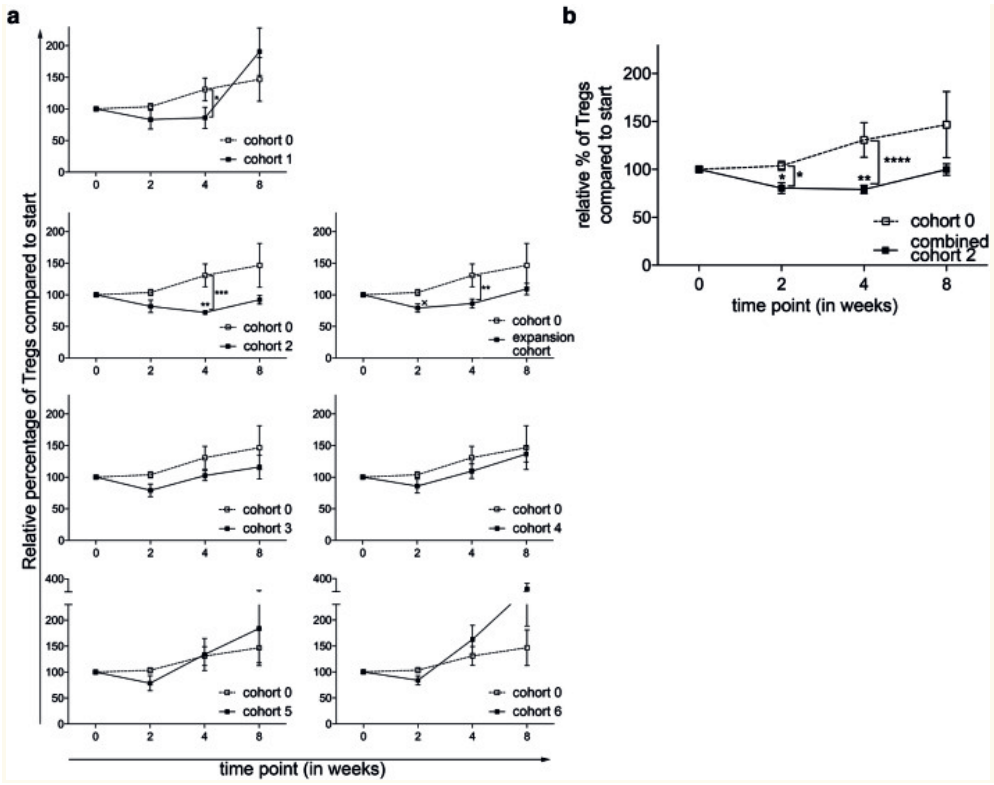

Figure 1. Effect of different dosages and administration schedules of CTX when combined with a fixed dose of 10 mg everolimus on the frequency of Tregs. **a** Relative percentages of Tregs within $CD4^+$ T cells were determined in freshly isolated PBMC from patients treated with different dosages and schedules of CTX, combined with a fixed dose of everolimus at baseline and subsequently 2, 4, and 8 weeks after start of treatment. *p* value indicated with asterisk; $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $^{x}p = 0.07$. **b** Relative percentages of Tregs within $CD4^+$ T cells are shown for cohort 2 combined with the expansion cohort. Patients were treated with 50 mg CTX once daily, combined with 10 mg everolimus once daily. Means ± SEM are shown; *p* value indicated with asterisk; $*p \leq 0.05$, $**p \leq 0.01$, $****$ $p \leq 0.0001$

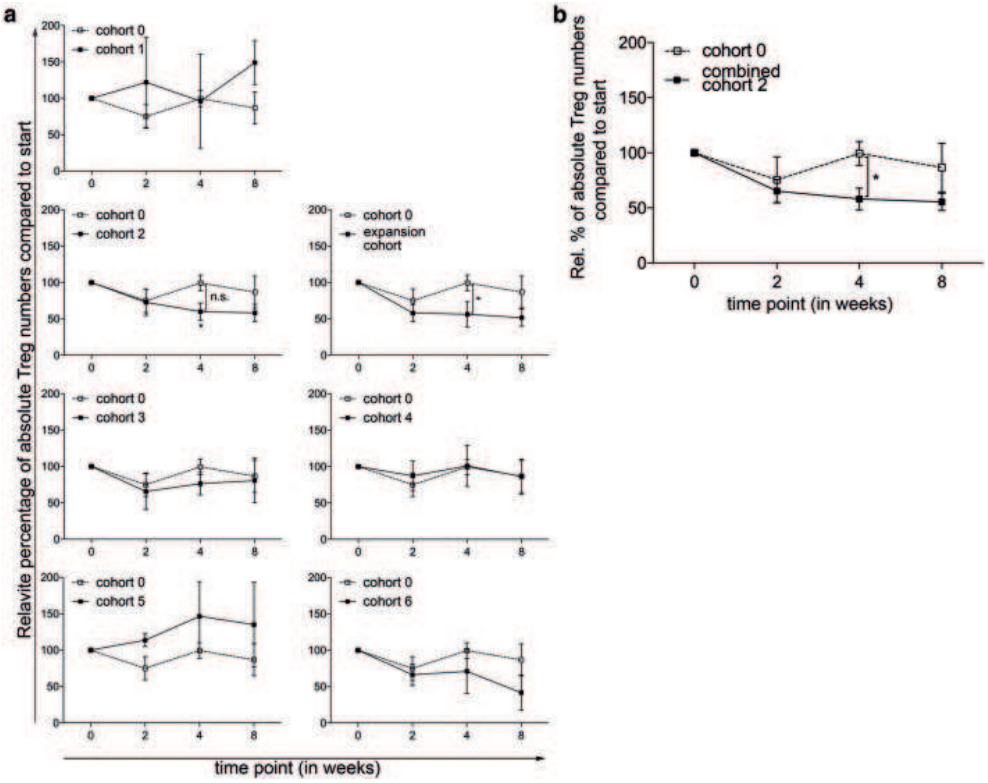

Figure 2. Effect of different dosages and administration schedules of CTX when combined with a fixed dose of 10 mg everolimus on absolute Treg numbers. **a** Relative percentages of absolute Treg numbers were determined in freshly isolated PBMC from patients treated with different dosages and schedules of CTX, combined with a fixed dose of everolimus at baseline and subsequently 2, 4, and 8 weeks after start of treatment. *p* value indicated with asterisk, $*p \leq 0.05$. **b** Relative percentages of absolute Treg numbers are shown for cohort 2 combined with the expansion cohort. Patients were treated with 50 mg CTX once daily, combined with 10 mg everolimus once daily. Means ± SEM are shown; *p* value indicated with asterisk; $* p \leq 0.05$

The expansion cohort essentially confirmed the results previously observed in cohort 2. Again, a decrease in Treg percentages was noted between time point 0 and 4 resulting in a statistically significant difference at this time point in Treg percentages between cohort 0 and the expansion cohort. When the results of cohort 2 and the expansion cohort were combined, a highly significant decrease in the percentage of Tregs was observed, both within the combined patient cohort as well as in comparison of this cohort to cohort 0 (Fig. 1b). In absolute Treg numbers the same decrease was observed in the expansion cohort, with a significant difference at timepoint 4 between cohort 0 and the expansion cohort. When absolute number data from cohort 2 were combined with those of the expansion cohort 2E, a significant decrease in absolute Treg numbers was noted (Fig. 2b).

Adverse events and DLT

During the entire study 314 adverse events were reported; 93 of these consisted of laboratory abnormalities (see Table 2 and supplementary table 2). The most common treatment-related toxicities (> 30%) included fatigue (*n* = 18; 46%), anorexia (*n* = 16; 41%), rash (*n* = 15; 38%), cough (*n* = 14; 36%), mucositis (*n* = 14; 36%), nausea (*n* = 12; 31%), anemia (*n* = 14; 36%), and hypercholesterolemia (*n* = 12; 31%). The mean number of adverse events of any grade was 8.2 per patient in the total group, while a mean of 5.4 adverse events per patient occurred in cohort 0 (i.e., in the cohort without CTX). When patients were treated for a longer period with the study drugs, more adverse events were reported. When adjusted, a mean of 3.2 adverse events per month was reported. After this adjustment, the two cohorts with the highest CTX dose showed slightly more adverse events compared to the lower cohorts. 47 treatment-related ≥ grade 3 toxicities were reported in 22 patients, and these consisted mainly of laboratory abnormalities (leukocytopenia, lymphocytopenia, hyperglycemia) and fatigue. One patient suffered from grade 4 lymphopenia after 10.5 months of treatment in cohort 5 in which 10 mg everolimus was combined with 100 mg CTX twice daily in a week-on/week-off schedule. A dose reduction had already taken place because of the toxicity, which had been present at a lower grade for a longer period. The grade 4 toxicity eventually lead to the decision to stop the study medication, followed by the radiological assessment of disease progression several days later.

Two patients experienced ≥ grade 3 toxicity within the first 28 days after start of the study treatment, one grade 3 pneumonitis and one grade 3 pancytopenia in combination with hyperglycemia. The patient with the grade 3 pneumonitis was treated in cohort 1, in which 10 mg of everolimus was combined with 50 mg CTX once daily in a week-on/week-off schedule. According to the protocol everolimus was interrupted resulting in improvement of the pneumonitis. Study medication was permanently discontinued and dyspnea persisted 46 days after the initiation of treatment and the patient showed radiological signs of progressive disease 10 days later. The patient with grade 3 pancytopenia in combination with hyperglycemia was treated in cohort 5, in which 10 mg everolimus was combined with 100 mg CTX twice daily in a week-on/week-off schedule. The adverse event occurred after 12 days of study drug administration and according to the protocol the treatment was temporarily stopped. Laboratory values improved and after 9 days of interruption both study drugs were restarted at half the original dose. Although both ≥ grade 3 toxicities occurred within the first 28 days from start of combination treatment, both occurred in different cohorts. Since ≤ 1 DLTs were experienced by the 5 patients in these cohorts, further patients could be enrolled at the next dose level.
Both in cohort 2, the cohort that showed a selective Treg depletion, as well as in the similarly dose expansion cohort 2E, three grade 3 adverse events were reported and no DLTs.

Table 2. Treatment-related toxicity

Event	Any Grade	Number of patients (%)		
		Grade 1	Grade 2	Grade ≥ 3
Neurology				
Neuropathy	4 (10)	3 (8)	1 (3)	0
Respiratory				
Cough	14 (36)	11 (28)	3 (8)	0
Dyspnea	10 (26)	5 (13)	4 (10)	1 (3)
Pneumonitis	7 (18)	1 (3)	3 (8)	3 (8)
Gastro-intestinal				
Mucositis	14 (36)	10 (26)	4 (10)	0
Nausea	12 (31)	6 (15)	6 (15)	0
Diarrhea	11 (28)	8 (20)	1 (3)	2 (5)
Vomiting	9 (23)	4 (10)	5 (13)	0
Dysgeusia	6 (15)	4 (10)	2 (5)	0
Stomatitis	5 (13)	3 (8)	1 (3)	1 (3)
Constipation	4 (10)	1 (3)	3 (8)	0
Renal/genitourinary				
(Hemorrhagic) cystitis	7 (18)	2 (5)	4 (10)	1 (3)
Pollakisuria	4 (10)	3 (8)	1 (3)	0
Constitutional				
Fatigue	18 (46)	5 (13)	8 (20)	5 (13)
Anorexia	16 (41)	8 (20)	8 (20)	0
Fever/chills/flu	5 (13)	5 (13)	0	0
Malaise	4 (10)	2 (5)	1 (3)	1 (3)
Dermatology				
Rash	15 (38)	9 (23)	6 (15)	0
Dry skin	8 (20)	6 (15)	2 (5)	0
Pruritus	4 (10)	4 (10)	0	0

Event	Any Grade	Number of patients (%)		
		Grade 1	Grade 2	Grade ≥ 3
Laboratory				
Anemia	14 (36)	2 (5)	10 (26)	2 (5)
Hypercholesterolemia	12 (31)	3 (8)	7 (18)	2 (5)
Lymphocytopenia	10 (26)	0	2 (5)	8 (20)
Hyperglycemia	10 (26)	1 (3)	6 (15)	3 (8)
Thrombocytopenia	10 (26)	7 (18)	1 (3)	2 (5)
Hypertriglyceridemia	8 (20)	3 (8)	3 (8)	2 (5)
Leukocytopenia	8 (20)	2 (5)	2 (5)	4 (10)
Electrolyte disturbance[a]	7 (18)	5 (13)	0	2 (5)
Liver values increased[b]	6 (15)	2 (5)	3 (8)	1 (3)
Neutropenia	5 (13)	0	3 (8)	2 (5)
Other				
Edema (extremities/face)	4 (10)	3 (8)	0	1 (3)

Reported in 10% or more of the treated patients

[a]Hypophosphatemia, hyponatremia, hypo- and hyperkalemia, hypocalcemia

[b]Alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase and alkaline phosphatase

VEGF levels

As chemotherapy was proposed to have anti-angiogenic effects in metronomic doses (reviewed in [28]), several studies showed decreased VEGF levels after treatment with metronomic CTX [29, 30]. For this study VEGF levels were measured at baseline, week 4 and (where available) week 8. The mean baseline VEGF level of all patients included in the study was 210 ± 30 pg/ml (mean ± SEM). As shown in supplementary Fig. 4, all cohorts in which patients received the combination treatment of everolimus and CTX showed lower VEGF levels during treatment as compared to cohort 0 in which patients received everolimus monotherapy. The cohorts with higher doses of CTX showed more pronounced effects; however, in neither of the cohorts, results were statistically significant.

Clinical outcome

The Overall Response Rate (ORR) did not significantly differ between the investigated cohorts. The best clinical response was a partial remission (PR) in 2 patients (5%); stable disease (SD) was observed in 22 patients (56%) and progressive disease (PD) in 15 patients (39%) (Fig. 3a). The responses per cohort are shown in Fig. 3b.

Median PFS among all cohorts was 3.5 months (range 1–24 months). At the end of the follow-up period 1 patient did not show progression, however, this patient stopped study treatment after 8 weeks due to toxicity. After 8.5 months this patient still did not show progression, and was lost to follow-up after 25 months. No significant differences in PFS were observed between the different cohorts. In Fig. 4 the PFS is shown per cohort. There was no statistically significant correlation between Treg numbers and PFS ($R = 0.01$, $p = 0.47$; data not shown).

Median OS was 11.5 months (range 1–45 months), 3 patients were still alive at the end of the follow-up period. No significant differences in OS were seen between the cohorts (see supplementary Fig. 5).

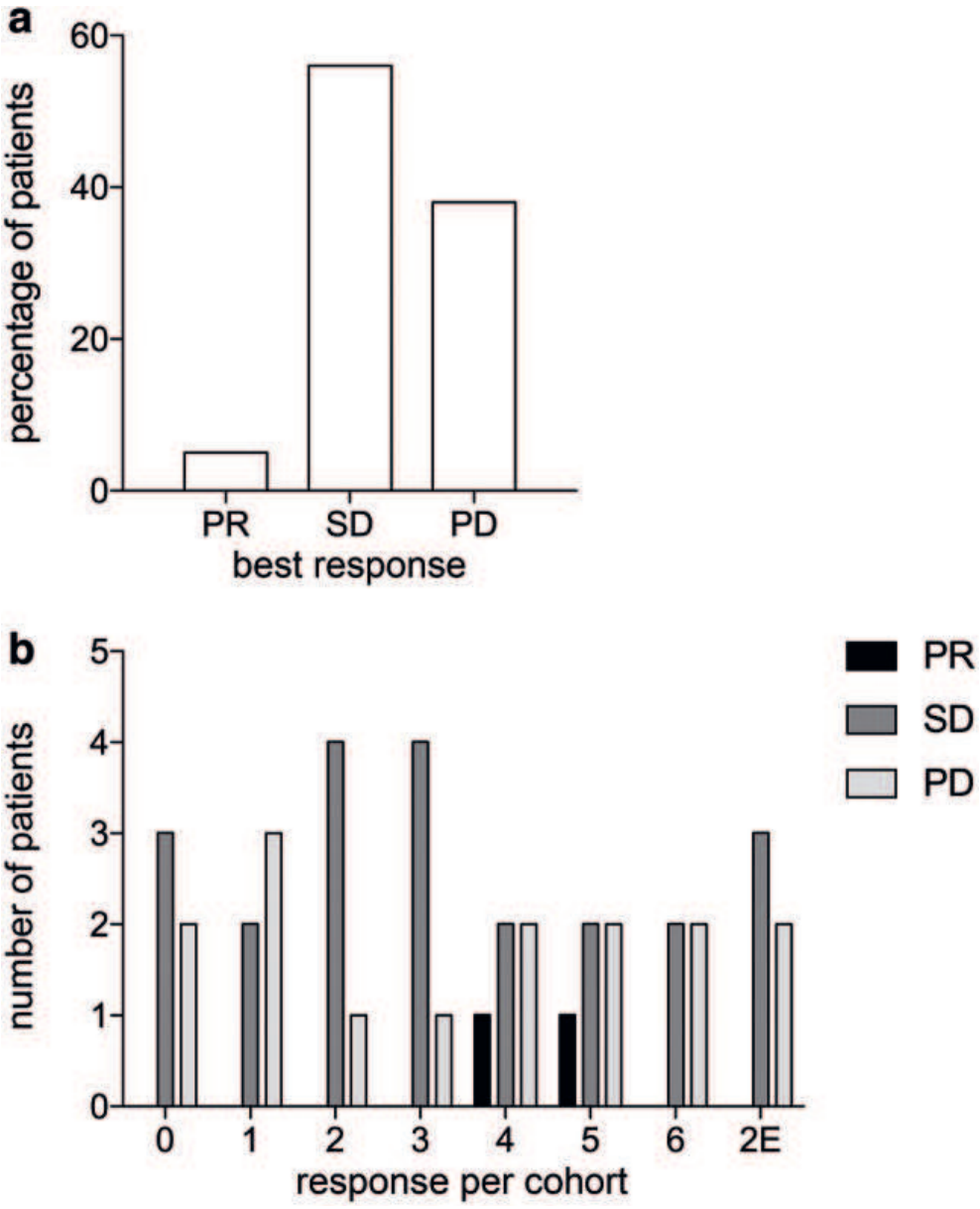

Figure 3. Clinical outcome.
A. Best clinical response for the total study population.
B. Best clinical response shown per cohort. Partial remission (PR) is shown in black, stable disease (SD) in grey and progressive disease (PD) in light grey.

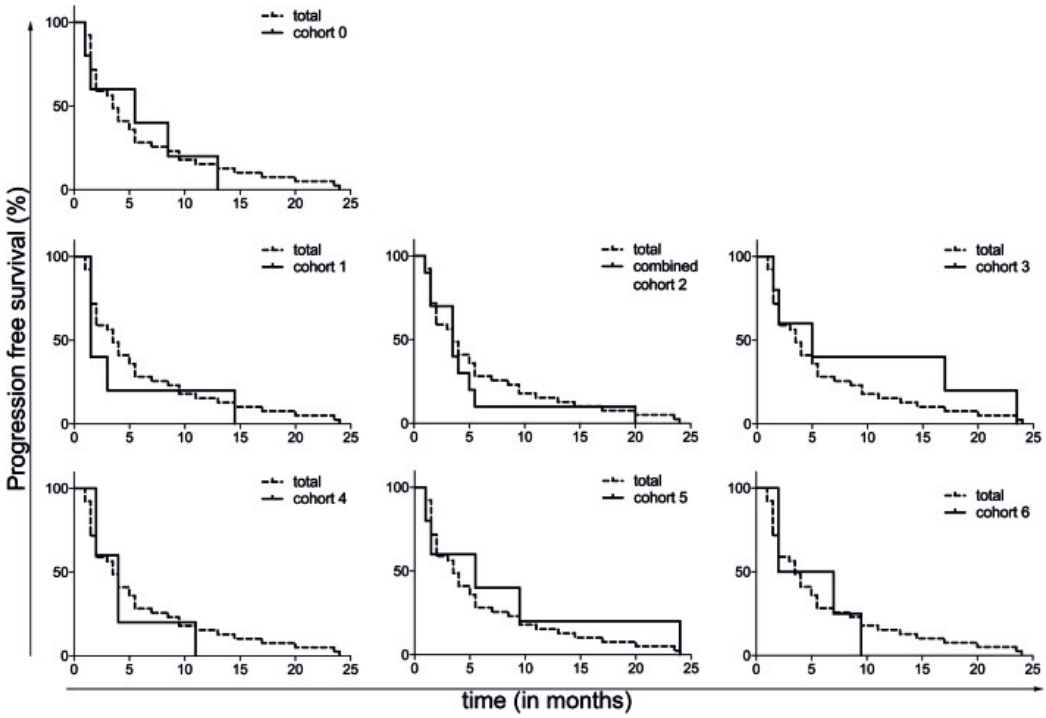

Figure 4. Kaplan–Meier curves for PFS per cohort, compared to the total patient group

Discussion

Since mTOR based regimens lead to Treg expansion [16–18] which can be considered an undesirable effect in the treatment of cancer, strategies that can selectively deplete Tregs might improve the antitumor effect of mTOR inhibitors by reversing the suppressive effect on the immune system. CTX was previously shown to result in selective Treg depletion [25, 26]; however, the optimal dose and schedule of metronomic CTX to induce selective Treg depletion in patients treated with mTOR inhibitors has not been determined. In the present trial, the Treg depleting effect of several dosages and schedules of metronomic CTX in combination with mTOR inhibition were investigated [27]. Our data indicate that a significant and selective Treg depletion in peripheral blood can be achieved when mRCC patients that receive the standard once daily oral dose of 10 mg everolimus are simultaneously treated with a once daily oral dose of 50 mg CTX, in a continuous scheme, whereas $CD4^+$ T cell percentages remain stable. The selected dose of CTX not only resulted in a significant decrease in the frequency of Tregs but also resulted in a significant decrease in absolute Treg numbers. Surprisingly, Treg percentages were found to actually increase when higher doses of CTX were administered. Since the exact mechanism responsible for Treg depletion is unknown, similarly this resistance of Tregs to higher CTX dosages remains unclear. Several mechanisms have been proposed to be responsible for the susceptibility of Tregs to CTX. For example, Tregs were shown (1) to have low ATP levels [31] leading to reduced synthesis of glutathione and thereby decreasing the detoxification of CTX, (2) to have DNA repair defects [32] due to high levels of DNA crosslinks and (3) to have deficient expression of ABCB1 [33] making them less able to extrude CTX. On the other hand, it was shown that Tregs express aldehyde dehydrogenase (ALDH), protecting them from CTX toxicity in graft-versus-host disease [34]. However, all those mechanisms cannot completely explain the observed effects, although it might be possible that Tregs acquire increased expression of ALDH, an effect that might be accelerated when higher dosages of cyclophosphamide are administered, possibly accounting for their apparent resistance to the depleting effects of CTX at these dose levels. Whether and which of these mechanisms may underlie the observed changes in the Treg population in the patients enrolled in this trial requires further investigation.

Across all the patient cohorts that were studied, we found that the combination of everolimus and CTX resulted in toxicity comparable to that observed in the RECORD-1 trial in patients with mRCC [35]. The toxicities that were observed in our trial were all known toxicities associated with both treatment regimens. The two observed DLTs, grade 3 pneumonitis in cohort 1 and grade 3 pancytopenia in combination with hyperglycemia in cohort 5, occurred in different cohorts, and therefore, did not affect further dose escalation of CTX. Common side effects of everolimus include lymphopenia, atypical infections, non-infectious pneumonitis and elevation of serum cholesterol, glucose, and triglycerides [36]. Although these adverse events were observed in this trial, the most common side effects were fatigue, anorexia, rash, cough, mucositis, nausea, anemia, and hypercholesterolemia. Though everolimus is a known causative drug for these side effects, we cannot exclude an additional effect of CTX. All adverse events could be alleviated by adjustment of the dose of the study drug or halting the study drug, and no deaths occurred due to the study medication. All cohorts were comparable with respect to the mean number of adverse events per patient, with a mean of 8.2 per patient. When patients were treated for a longer period with the study drugs, more adverse events were reported. The two cohorts with the highest CTX dose showed slightly more adverse events compared to the lower cohorts. Interestingly, addition of CTX to everolimus resulted in lower VEGF levels compared to the cohort in which single everolimus treatment was administered. These results were not statistically significantly different, probably due to small sample sizes and missing values at timepoint 8 weeks.

As secondary endpoints, the ORR, and median PFS and OS were calculated. Since the cohorts were small, only 5 patients per cohort, the survival data were calculated for all patients combined as shown in Fig. 4 and supplementary Fig. 5, and additionally shown for all cohorts separately. While the phase 2 part of the trial will allow formal assessment of the effect of the addition of the selected once daily oral dose of 50 mg of CTX on the clinical efficacy of everolimus, the data presented here at least show no sign of inferiority compared to historical results of everolimus monotherapy in mRCC.

In conclusion, in this trial we demonstrate that administration of 50 mg CTX once daily in a continuous schedule leads to depletion of Tregs when combined with 10 mg everolimus once daily, with toxicity comparable to that reported in the RECORD-1 trial.

The treatment combination is currently under investigation in a phase 2 trial, to determine if the observed Treg depletion also results in an enhancement of the survival of patients with mRCC when compared to everolimus alone. Recently everolimus was replaced by both nivolumab and cabozantinib as the standard second line treatment for patients with mRCC [7]. In case the phase 2 part of the trial shows beneficial effects on survival, combination therapy of CTX and everolimus could still be implemented in a later treatment line. However, when everolimus is combined with lenvatinib the additional effect of CTX might be limited as, e.g. the tyrosine kinase inhibitor sunitinib, that like lenvatinib inhibits VEGF and other receptors [37, 38], was previously shown to decrease Treg frequencies [39, 40]. Besides, a sequential treatment schedule of everolimus and cyclophosphamide could be proposed, which might result in reduced Treg levels with less toxicity. Since CTX is a well-known and broadly used drug, there is much experience in the application of this drug. In addition, it is cheap, which is an advantage especially when compared to the cost of recently developed novel therapeutics. Furthermore, since everolimus is registered for the treatment of pancreatic neuroendocrine tumors, these patients might also benefit from the same treatment combination [41].

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Supplementary table 1. Blood measurements

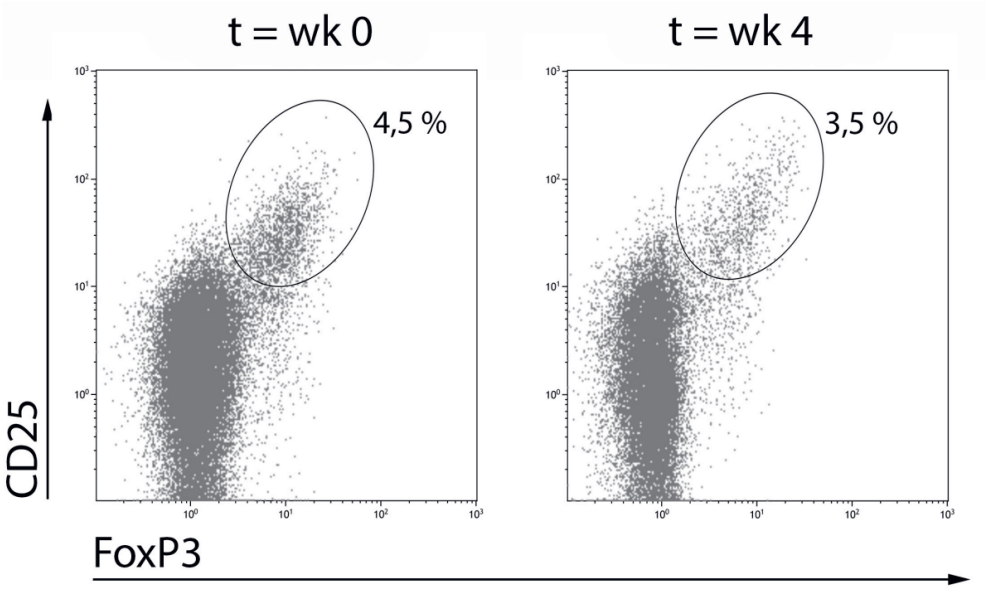
Measurements	Value (range)			
	Baseline	t=2	t=4	t=8
Hemoglobin (mmol/L)	7,5 (5,2-10,3)	7,2 (5,3-9,2)	6,8 (4,8-9,2)	6,5 (5,2-9,2)
WBC (x 10^9/L)	6,7 (2,9-11,1)	4,7 (2,2-7,5)	5,4 (1,8-12,3)	5,2 (1,4-13,4)
Neutrophils (x 10^9/L)	4,4 (1,7-9,6)	3 (1,3-6,2)	3,6 (1,1-8,5)	3,7 (0,9-12,1)
Eosinophils (x 10^9/L)	0,1 (0-0,5)	0,2 (0,05-0,4)	0,16 (0,03-0,62)	0,2 (0-0,64)
Basophils (x 10^9/L)	0,05 (0-0,1)	0,05 (0-0,1)	0,06 (0-0,18)	0,05 (0-0,1)
Lymphocytes (x 10^9/L)	1,5 (0,5-4,2)	1,1 (0,4-3,1)	1 (0,3-2,23)	0,8 (0,09-2,1)
Monocytes (x 10^9/L)	0,61 (0,2-1,15)	0,4 (0,1-0,8)	0,5 (0,2-1,47)	0,5 (0,02-1,15)
Platelets (x 10^9/L)	286 (121-585)	197 (47-528)	273 (85-691)	256 (76-684)
Corrected Calcium (mmol/L)	2,5 (2,2-3)	2,4 (2,1-2,7)	2,5 (2,2-3,3)	2,4 (2,1-3,0)
LDH (U/L)	250 (80-2133)	240 (80-1734)	341 (107-2614)	263 (113-454)

Supplementary table 2. Treatment-related toxicity per cohort

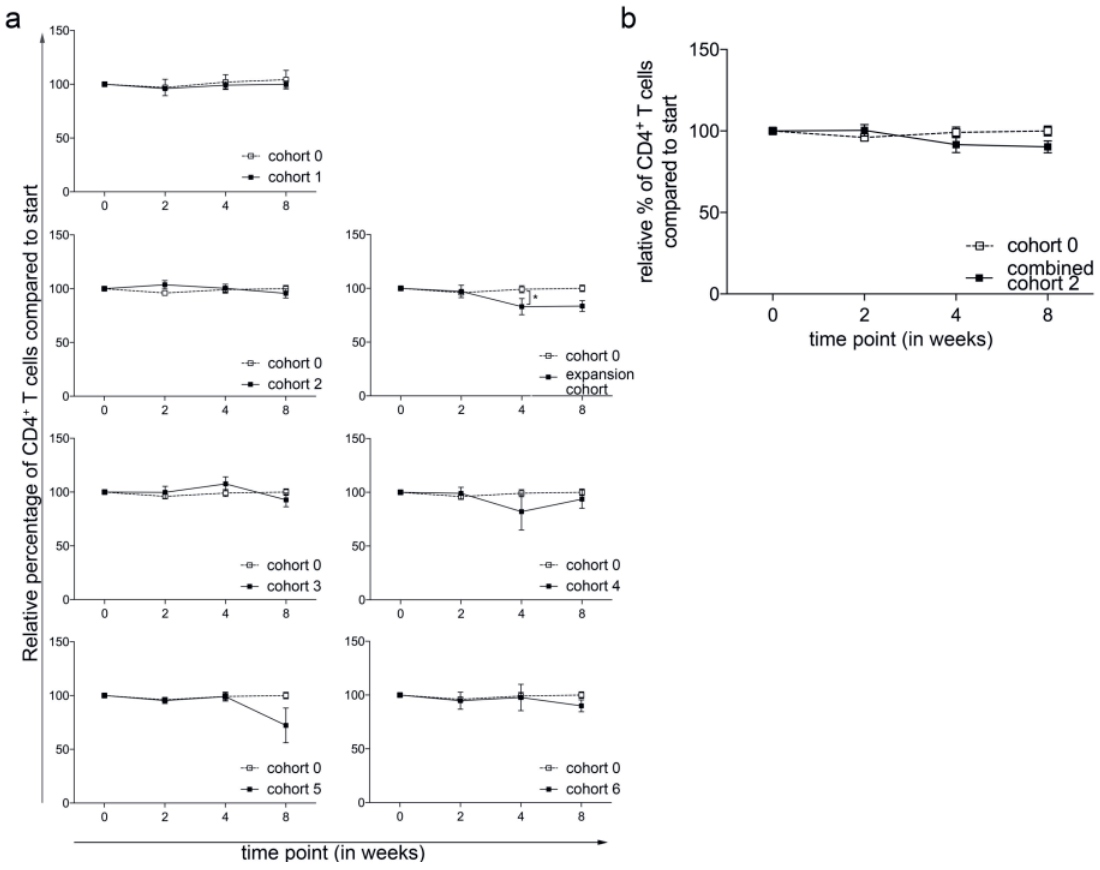
Events	All cohorts	Cohort 0	Cohort 1	Cohort 2	Cohort 3	Cohort 4	Cohort 5	Cohort 6	Cohort 2E
				Number of patients					
Cough									
Any Grade	**14 (36%)**		**3**	**4**	**1**		**3**	**2**	**1**
Grade 1	11 (28%)		2	3	1		2	2	1
Grade 2	3 (8%)		1	1			1		
Dyspnea									
Any Grade	**10 (26%)**	**1**	**3**	**2**			**1**	**2**	**1**
Grade 1	5 (13%)	1	1	2					1
Grade 2	4 (10%)		1			1		2	
Grade ≥3	1 (3%)		1						
Pneumonitis									
Any Grade	**7 (18%)**		**3**	**2**	**1**		**1**		
Grade 1	1 (3%)			1					
Grade 2	3 (8%)		1	1	1				
Grade ≥3	3 (8%)		2			1			
Mucositis									
Any Grade	**14 (36%)**	**1**	**1**	**1**	**3**	**2**	**1**	**2**	**3**
Grade 1	10 (26%)	1		1		2	1	2	3
Grade 2	4 (10%)		1		3				
Nausea									
Any Grade	**12 (31%)**	**1**	**1**			**3**	**2**	**2**	**3**
Grade 1	6 (15%)		1			2		1	2
Grade 2	6 (15%)	1				1	2	1	1
Diarrhea									
Any Grade	**11 (28%)**	**2**	**2**		**1**	**1**	**1**	**1**	**3**
Grade 1	8 (20%)	1	2		1			1	3
Grade 2	1 (3%)	1							
Grade ≥3	2 (5%)					1	1		
Vomiting									
Any Grade	**9 (23%)**	**1**	**1**			**2**	**2**	**2**	**1**
Grade 1	4 (10%)		1			1	1	1	
Grade 2	5 (13%)	1				1	1	1	1
Dysgeusia									
Any Grade	**6 (15%)**	**1**		**2**			**1**	**1**	**1**
Grade 1	4 (10%)	1		1			1		1
Grade 2	2 (5%)			1				1	
Stomatitis									
Any Grade	**5 (13%)**		**1**	**2**	**1**	**1**			
Grade 1	3 (8%)			1	1	1			
Grade 2	1 (3%)		1						
Grade ≥3	1 (3%)			1					
Constipation									
Any Grade	**4 (10%)**							**1**	**3**
Grade 1	1 (3%)								1
Grade 2	3 (8%)							1	2
(Hemorrhagic) cystitis									
Any Grade	**7 (18%)**			**1**		**1**	**2**	**2**	**1**
Grade 1	2 (5%)						1		1
Grade 2	4 (10%)			1		1	1	1	
Grade ≥3	1 (3%)							1	
Pollakiuria									
Any Grade	**4 (10%)**					**1**		**2**	**1**
Grade 1	3 (8%)							2	1
Grade 2	1 (3%)					1			
Fatigue									
Any Grade	**18 (46%)**	**2**	**2**	**4**	**1**		**3**	**2**	**4**
Grade 1	5 (13%)		2	2			1		
Grade 2	8 (20%)	1		2	1		1	1	2
Grade ≥3	5 (13%)	1		1			1	1	2
Anorexia									
Any Grade	**16 (41%)**	**3**	**1**	**2**		**4**	**1**	**2**	**3**
Grade 1	8 (20%)	2	1	1		2	1		1
Grade 2	8 (20%)	1		1		2		2	2
Fever/ chills/flu									
Any Grade	**5 (13%)**			**2**	**2**	**1**			
Grade 1	5 (13%)			2	2	1			
Malaise									
Any Grade	**4 (10%)**	**1**		**1**					**2**
Grade 1	2 (5%)	1							1
Grade 2	1 (3%)			1					
Grade ≥3	1 (3%)								1

Events	All cohorts	Cohort 0	Cohort 1	Cohort 2	Cohort 3	Cohort 4	Cohort 5	Cohort 6	Cohort 2E
	Number of patients								
Pruritus									
Any Grade	**4 (10%)**				**2**		**2**		
Grade 1	4 (10%)				2		2		
Anemia									
Any Grade	**14 (36%)**	**1**	**1**	**3**	**1**	**1**	**3**	**2**	**2**
Grade 1	2 (5%)	1	1						
Grade 2	10 (26%)			2	1	1	2	2	2
Grade ≥3	2 (5%)			1			1		
Hypercholesterolemia									
Any Grade	**12 (31%)**	**1**	**1**	**2**	**4**	**1**		**1**	**2**
Grade 1	3 (8%)				1				2
Grade 2	7 (18%)	1		2	2	1		1	
Grade ≥3	2 (5%)		1		1				
Lymphocytopenia									
Any Grade	**10 (26%)**		**1**		**1**	**2**	**3**	**3**	
Grade 2	2 (5%)						1	1	
Grade ≥3	8 (20%)	1			1	2	2	2	
Hyperglycemia									
Any Grade	**10 (26%)**	**1**	**2**	**2**		**1**	**2**	**1**	**1**
Grade 1	1 (3%)								1
Grade 2	6 (15%)	2		2		1		1	
Grade ≥3	3 (8%)	1					2		
Thrombocytopenia									
Any Grade	**10 (26%)**		**1**	**1**	**3**	**2**	**2**		**1**
Grade 1	7 (18%)		1	1	2	1	1		1
Grade 2	1 (3%)				1				
Grade ≥3	2 (5%)					1	1		
Hypertriglyceridemia									
Any Grade	**8 (20%)**	**1**	**2**	**1**	**2**		**1**		**1**
Grade 1	3 (8%)	1	1		1				
Grade 2	3 (8%)			1			1	1	
Grade ≥3	2 (5%)		1		1				
Leukocytopenia									
Any Grade	**8 (20%)**			**1**	**1**	**1**	**1**	**1**	**3**
Grade 1	2 (5%)								2
Grade 2	2 (5%)			1					1
Grade ≥3	4 (10%)				1	1	1	1	
*Electrolyte disturbance**									
Any Grade	**7 (18%)**	**2**				**1**		**1**	**3**
Grade 1	5 (13%)	2							3
Grade ≥3	2 (5%)					1		1	
*Liver values increased***									
Any Grade	**6 (15%)**		**1**		**1**	**1**	**1**	**2**	
Grade 1	2 (5%)							2	
Grade 2	3 (8%)		1			1	1		
Grade ≥3	1 (3%)				1				
Neutropenia									
Any Grade	**5 (13%)**				**1**	**1**	**1**	**1**	**1**
Grade 2	3 (8%)						1	1	1
Grade ≥3	2 (5%)				1	1			
Edema (extremities/ face)									
Any Grade	**4 (10%)**	**1**			**1**	**1**		**1**	
Grade 1	3 (8%)	1			1		1		
Grade ≥3	1 (3%)					1			
Dry skin									
Any Grade	**8 (20%)**			**1**	**3**	**2**			**1**
Grade 1	6 (15%)			1	1	2	1		1
Grade 2	2 (5%)					2			
Rash									
Any Grade	**15 (38%)**	**1**	**1**	**2**	**4**	**2**	**2**	**1**	**2**
Grade 1	9 (23%)			1	2	2	1	1	2
Grade 2	6 (15%)	1	1	1	2		1		
Neuropathy									
Any Grade	**4 (10%)**	2	3			3			
Grade 1	3 (8%)	1	1			1			
Grade 2	1 (3%)	1							

Reported in 10% or more of the treated patients
** Hypophosphatemia, hyponatremia, hypo- and hyperkalemia, hypocalcemia*
*** Alanine aminotransferase, aspartate aminotransferase, gamma- glutamyl transferase and alkaline phosphatase*



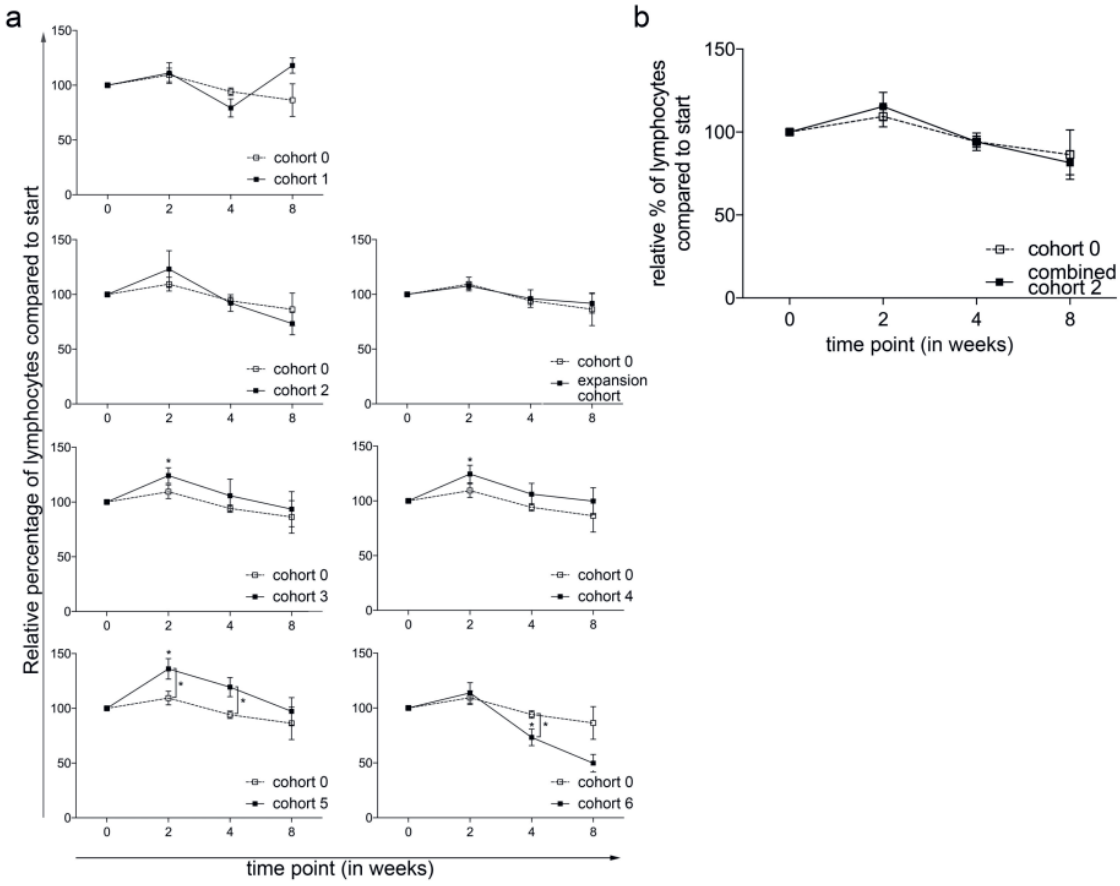
Supplementary fig. 1. Representative flow cytometry dot plots illustrating the changes in Tregs, defined as $CD3^{+}CD4^{+}CD25^{hi}FoxP3^{+}$



Supplementary fig. 2. Effect of different dosages and administration schedules of CTX when combined with a fixed dose of 10 mg everolimus on the frequency of $CD4^+$ T cells.

A. Relative percentages of $CD4^+$ T cells within $CD3^+$ T cells were determined in freshly isolated PBMC from patients treated with different dosages and schedules of CTX, combined with a fixed dose of everolimus at baseline and subsequently 2, 4, and 8 weeks after start of treatment.

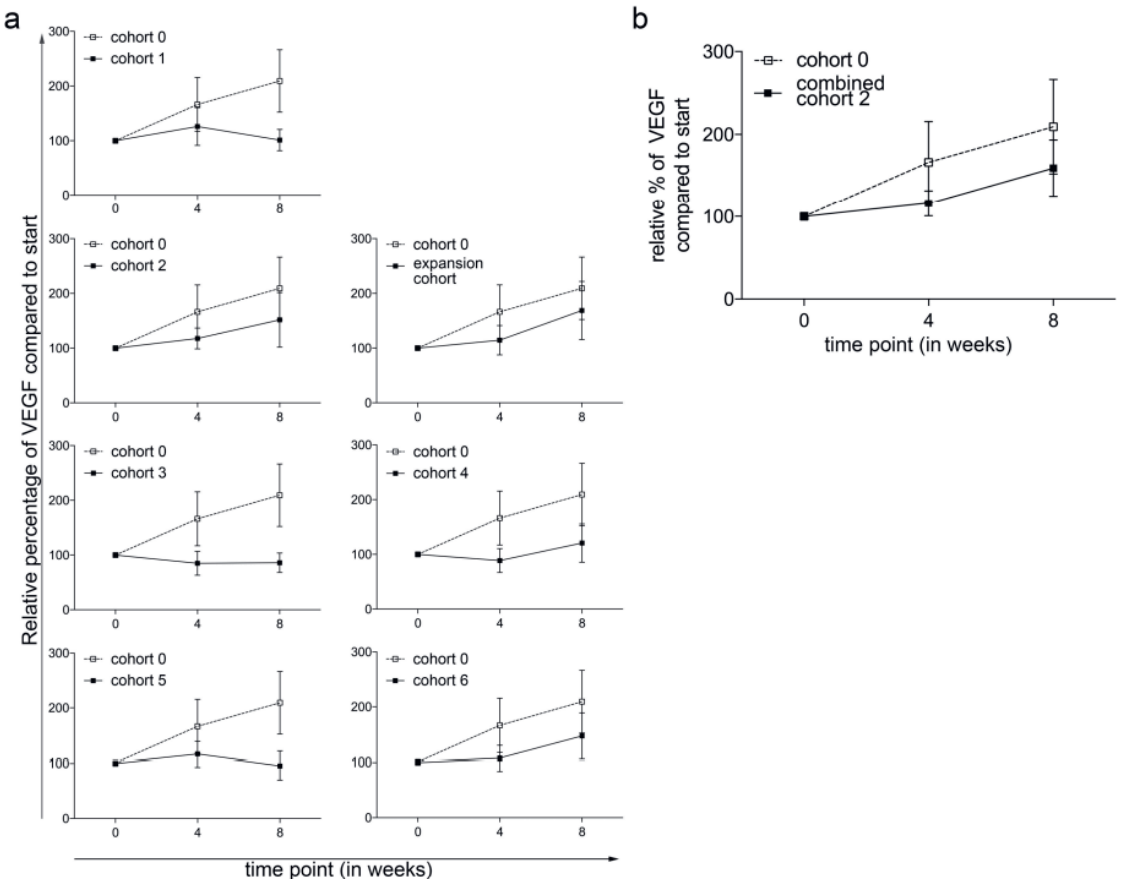
B. Relative percentages of $CD4^+$ T cells within $CD3^+$ T cells are shown for cohort 2 combined with the expansion cohort. Patients were treated with 50 mg CTX once daily, combined with 10 mg everolimus once daily. Means ± SEM are shown; p-value indicated with asterisk; * $p \leq 0.05$.



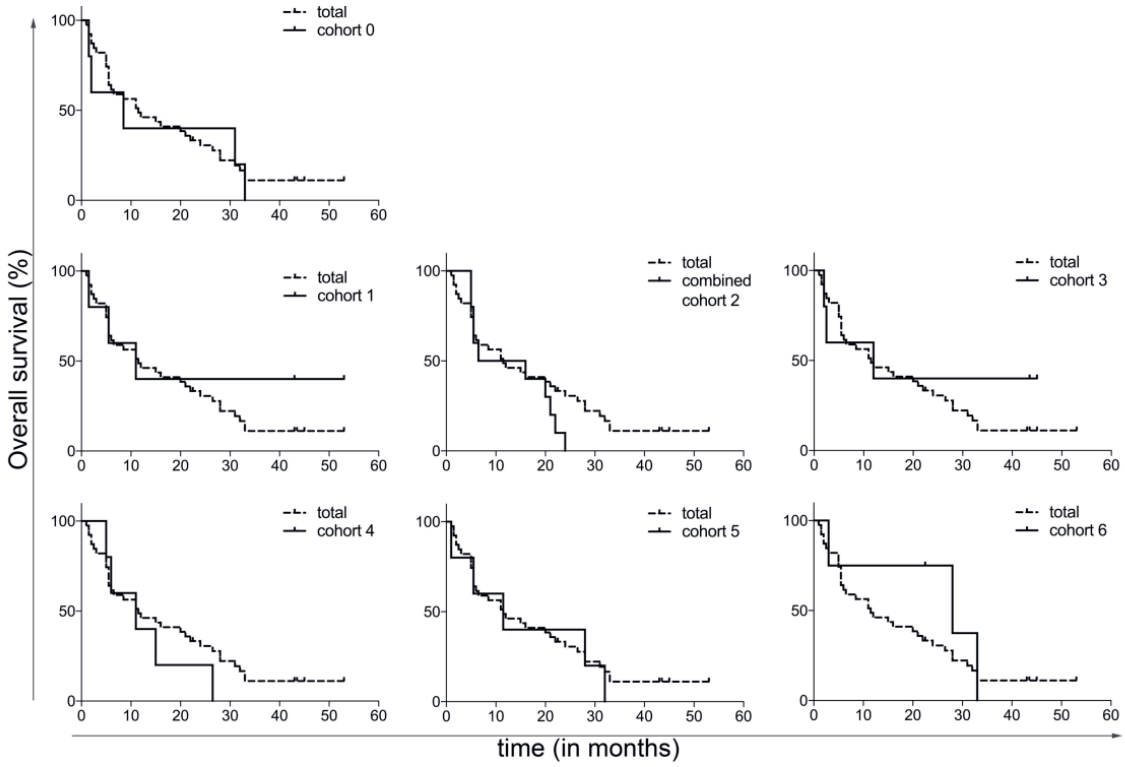
Supplementary fig. 3. Effect of different dosages and administration schedules of CTX when combined with a fixed dose of 10 mg everolimus on the frequency of lymphocytes.

A. Relative percentages of lymphocytes were determined in freshly isolated PBMC from patients treated with different dosages and schedules of CTX, combined with a fixed dose of everolimus at baseline and subsequently 2, 4, and 8 weeks after start of treatment.

B. Relative percentages of lymphocytes (within PBMC) are shown for cohort 2 combined with the expansion cohort. Patients were treated with 50 mg CTX once daily, combined with 10 mg everolimus once daily. Means ± SEM are shown; p-value indicated with asterisk; * p≤ 0.05.



Supplementary fig. 4. Effect of different dosages and administration schedules of CTX when combined with a fixed dose of 10 mg everolimus on VEGF levels in plasma.
A. VEGF levels were determined in heparin plasma at baseline and subsequently 4, and 8 weeks after start of treatment. Here, relative percentages are shown. Baseline VEGF levels (mean ± SD) per cohort: cohort 0 – 286 ± 193 pg/mL, cohort 1 – 255 ± 128 pg/mL, cohort 2 – 139 ± 104 pg/mL, cohort 3 – 122 ± 17 pg/mL, cohort 4 – 217 ± 65 pg/mL, cohort 5 – 362 ± 113 pg/mL, cohort 6 – 174 ± 27 pg/mL
B. Relative percentages of VEGF plasma levels are shown for cohort 2 combined with the expansion cohort. Patients were treated with 50 mg CTX once daily, combined with 10 mg everolimus once daily. Mean VEGF level was 133 ± 25 pg/mL.
Means ± SEM are shown. No significant changes were observed.



Supplementary fig. 5. Kaplan-Meier curves for OS per cohort, compared to the total patient group.

Chapter 3 Metronomic cyclophosphamide attenuates mTOR-mediated expansion of regulatory T cells, but does not impact clinical outcome in patients with metastatic renal cell cancer treated with everolimus

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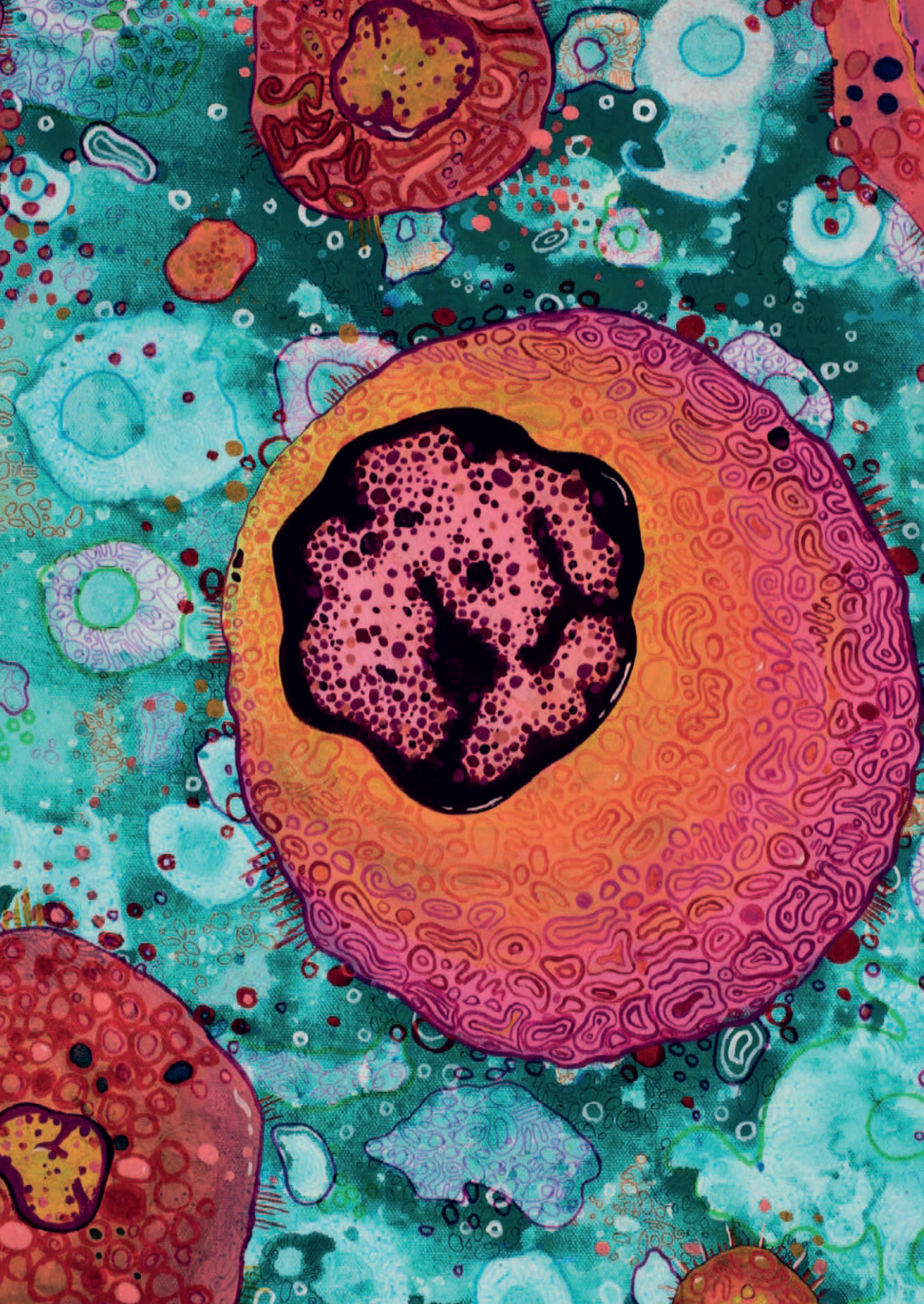


Abstract

Introduction: Metastatic renal cell cancer (mRCC) patients have a median overall survival (mOS) of approximately 28 months. Until recently, mammalian target of rapamycin (mTOR) inhibition with everolimus was the standard second-line treatment regimen for mRCC patients, improving median progression-free survival (mPFS). Treatment with everolimus supports the expansion of immunosuppressive regulatory T cells (Tregs), which exert a negative effect on antitumor immune responses. In a phase 1 dose-escalation study, we have recently demonstrated that a low dose of 50 mg oral cyclophosphamide once daily can be safely combined with everolimus in mRCC patients and prevents the everolimus-induced increase in Tregs.

Materials and methods: In a multicenter phase 2 study, performed in patients with mRCC not amenable to or progressive on a vascular endothelial growth factor (VEGF)-receptor tyrosine kinase inhibitor (TKI) containing treatment regimen, we assessed whether the addition of this metronomic dosing schedule of cyclophosphamide to therapy with everolimus could result in an improvement of progression-free survival (PFS) after 4 months of treatment.

Results: Though results from this study confirmed that combination treatment effectively lowered circulating levels of Tregs, addition of cyclophosphamide did not improve the PFS rate at 4 months. For this reason, the study was abrogated at the predefined interim analysis.

Conclusion: Although the comprehensive immunomonitoring analysis performed in this study provides relevant information for the design of future immunotherapeutic approaches, the addition of metronomic cyclophosphamide to mRCC patients receiving everolimus cannot be recommended.

Introduction

Renal cell cancer (RCC) has been diagnosed in more than 84.000 new patients in the European Union each year and has resulted in almost 34.000 cancer deaths in 2012 [1]. Death due to RCC is mostly a consequence of metastatic disease, which occurs in 30% of patients at presentation and in an additional 30% of patients after initial nephrectomy [2]. Metastatic RCC (mRCC) is known to be resistant to chemotherapy. However, the prognosis of mRCC has greatly improved in the last decade with the registration of various novel therapeutics, resulting in a current median overall survival (mOS) of 28–29 months [3,4,5,6]. New drugs have been mostly tested in patients with clear cell mRCC, while papillary, chromophobic and oncocytic RCC and RCC of the collecting duct have been studied less due to their lower prevalence [7]. Until recently, first-line treatment of clear cell mRCC patients predominantly consisted of drugs that block the intracellular domain of the vascular endothelial growth factor (VEGF) receptor, such as sunitinib or pazopanib, resulting in a median progression-free survival (mPFS) of 11 months [8,9,10], or the combination of interferon-alfa (IFN-α) and bevacizumab, the latter binding circulating VEGF, which resulted in an mPFS of 8.5–10 months [11,12,13,14]. Since 2007, drugs targeting the mammalian target of rapamycin (mTOR) pathway have been registered for the treatment of mRCC. Temsirolimus represents a first-line treatment option in poor-risk mRCC patients, while everolimus became a standard Food and Drug Administration (FDA)-approved second-line treatment in 2009 [10, 15,16,17,18]. The mTOR pathway influences cell growth, proliferation and angiogenesis, and mTOR inhibitor everolimus leads to an mPFS of approximately 4 months when used as second-line treatment [16, 19]. Recently, the programmed cell death protein-1 (PD-1) checkpoint-inhibitor nivolumab, the c-Met and VEGF tyrosine kinase inhibitor (TKI) cabozantinib and the combination of lenvatinib (a multi kinase inhibitor) and everolimus were shown to be more effective compared to everolimus monotherapy and have thereby replaced everolimus as the standard second-line therapeutic approach in mRCC patients [3, 4, 20, 21]. In addition, combination therapy with PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) immune checkpoint inhibitors nivolumab and ipilimumab was approved as a first-line treatment option for intermediate- and poor-risk patients [22].

One aspect potentially limiting the antitumor effect of mTOR inhibition by everolimus is their known stimulatory effect on regulatory T cells (Tregs) [23, 24]. Tregs are characterized by the expression of CD4, CD25 and the transcription factor forkhead box P3 (Foxp3), and are known to exert immunosuppressive effects, which can be beneficial in preventing overt autoimmunity, but can hamper the development of antitumor immune responses. Tumor cells or tumor-associated macrophages can produce ligands that selectively attract Tregs through C–C chemokine receptor (CCR) type 4, facilitating tumor cells to escape antitumor immunity [25, 26]. Studies have shown that the frequency of circulating as well as (peri)tumoral Tregs is negatively associated with survival in cancer patients, including mRCC patients [27,28,29].
We and others have shown that treatment with everolimus resulted in an expansion of peripheral blood Tregs [30, 31]. As we hypothesized that the undesirable everolimus-induced expansion of Tregs in mRCC patients could be counteracted, we co-administered cyclophosphamide, which is an alkylating agent of the nitrogen mustard type that is known to selectively deplete Tregs (and not helper or cytotoxic T cells) [32,33,34]. The effect of cyclophosphamide on Tregs is not completely understood; however, several mechanisms have been proposed, including (a) induction of a DNA repair defect, (b) reduction of the ATP and glutathion content of Tregs and (c) causing a lack in the expression of the ATP-binding cassette (ABC) transporters B1 (ABCB1) [33,34,35,36].
We first performed a phase 1 dose-escalation trial, in which we established the optimal dose of metronomic cyclophosphamide that, when combined with the standard once daily oral dose of 10 mg of everolimus, was safe, well tolerated and effectively reduced circulating levels of Tregs [37, 38]. In the present phase 2 study, we investigated whether the addition of the selected dose of metronomic cyclophosphamide would result in an improvement in mPFS as compared to everolimus monotherapy. In addition, immunomonitoring was performed to evaluate whether immune effects could be related to clinical outcome. The immunomonitoring performed in this study gives insight into the effects of mTOR inhibition and low-dose oral cyclophosphamide in cancer patients and thereby provides relevant information for the design of future treatments that incorporate or are based on mTOR inhibitors.

Patients and treatment

The multicenter study was performed in medical centers that were part of the WIN-O (The Working group Immunotherapy of the Netherlands for Oncology) and included 29 patients of 18 years or older with clear cell mRCC who were not amenable to, or had progressed on, a VEGF receptor TKI regimen. As originally planned in the design of the study, 10 of the 25 patients had participated in the phase 1 part of this study, where they had been treated with the same treatment regimen as in the here reported phase 2 study. For a more extensive description of the inclusion and exclusion criteria of the study, we refer to the published study protocol [39]. Follow-up was until death or until the time of analysis (9 months after inclusion of the last patient). A pre-planned interim analysis was performed after 24 patients were treated for at least 4 months, to assess whether the primary objective, an increase of progression-free survival (PFS) at 4 months from 50 to 70% could be achieved. Since 12 out of 24 patients had progressed within the first 4 months of treatment, the study was terminated prematurely due to lack of efficacy. Secondary objectives that were studied included response rate, time to progression, overall survival and an assessment of the immunological effects of combination treatment.

Patients were treated with 10 mg everolimus and 50 mg cyclophosphamide orally, both once daily continuously. In case of severe toxicity, dose reductions were allowed according to protocol. Adverse events (AE) were defined in accordance with the International Conference on Harmonisation (ICH) Guideline for Good Clinical Practice (ICH E6:1.2). Severity of clinical AE was graded according to the National Cancer Institute Common Toxicity Criteria (CTC) grading system version 3.0 (NCI-CTCAE). Dose-limiting toxicities (DLT) were toxicities attributable to combination therapy within the first 28 days of therapy and defined as febrile neutropenia, neutropenic infection, other grade ≥ 3 hematological toxicity, pneumonitis, nausea, vomiting, diarrhea, fatigue or any other grade ≥ 3 AE that, despite appropriate supportive care, failed to recover to grade ≤ 1 within 7 days [39]. Patients were evaluated at baseline and then every 4 weeks until the end of study treatment by means of history, physical examination and laboratory evaluation (hematology and chemistry).

Moreover computed tomography scans (CT scan) of chest and abdomen were made at baseline and thereafter every 8 weeks. The objective response rate (ORR) was assessed clinically and radiologically, using Response Evaluation Criteria In Solid Tumors (RECIST, version 1.1).

Immunomonitoring

Immunomonitoring was performed at baseline and 4 weeks after the start of the study treatment period. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Axis-Shield, Oslo, Norway) density-gradient centrifugation, cryopreserved in liquid nitrogen, thawed and subsequently stained for 30 min at 4 °C with labeled antibodies in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide. Based on the immunomonitoring results of the previously performed phase 1 study [37, 38], the following immune cell subsets were selected for monitoring in the present phase 2 study: regulatory T cells (Tregs, $CD4^+CD25^{hi}FoxP3^{hi}$), cytotoxic T cells (CTL, $CD3^+CD8^+$), B lymphocytes ($CD19^+$), myeloid dendritic cells (cDC1, $BDCA3^+CD14^-CD11c^+$ and cDC2, $BDCA1^+CD19^-CD14^-CD11c^+$) and plasmacytoid dendritic cells (pDC, $BDCA2^+CD11c^-CD123^+$), immunoregulatory ($CD56^{bright}CD16^{dim}$) and cytotoxic ($CD56^{dim}CD16^+$) natural killer cells (NK), and granulocytic ($Lin^-CD14^-CD33^+HLA^-DR^-$) and monocytic ($Lin^-CD14^+HLA^-DR^-$) myeloid derived suppressor cells (MDSC).
The following antibodies were used: FITC-labeled antibodies against IgG1, CD4, CD14, CD16, BDCA1, BDCA2 and BDCA3; PE-labeled antibodies against IgG1, CD8, CD19, CD40, CD56, CD86 and CD123; PerCP-labeled antibodies against IgG1, CD3 and CD4; APC-labeled antibodies against IgG1, CD3, CD11c, CD25 and PD-1 (all these antibodies were obtained from BD Biosciences, New Jersey, USA). Intracellular stainings were performed after fixation and permeabilization using a fixation/permeabilization kit according to the manufacturer's protocol (eBioscience, Massachusetts, USA). The labeled antibodies used for intracellular stainings were PE-labeled IgG1, IgG2a, CTLA-4 and Ki-67 (all BD Biosciences, New Jersey, USA). FoxP3 was stained with anti-FoxP3 mAbs, either PCH101 PE (eBioscience, Massachusetts, USA) or 259D Alexa Fluor 488 (Biolegend, San Diego, USA). All cells were analyzed on a BD FACS Calibur and analyzed using Kaluza Analysis Software (Beckman Coulter).

Statistical analysis

Paired *t* tests were used to determine the statistical significance of differences between time points or groups. PFS was defined as the time from baseline until progression or death. Overall survival (OS) was defined as the time from baseline until death. Both PFS and OS were analyzed using Kaplan–Meier curves. Correlations were measured using Pearson correlation coefficient. Findings were considered statistically significant when p values were ≤ 0.05, as indicated with asterisks ($^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Statistical analyses were performed using GraphPad Prism 6.0 software.

Results

Baseline patient characteristics

The study included 29 patients with clear cell mRCC who were treated at 12 different centers in the Netherlands between November 2013 and October 2016. Of these 29 patients, 25 patients were followed according to protocol; 4 of 29 patients were excluded within the first 2 weeks of the start of treatment. Three of them withdrew consent and one patient had inadvertently taken an inappropriate dose of cyclophosphamide. Patient characteristics are shown in Table 1. Of the 25 patients included for study analysis, 60% were male, the median age of the study group was 66 years and 80% could be categorized in the favorable or intermediate IMDC risk group (prognostic model according to the International Metastatic Renal Cell Carcinoma Database Consortium, IMDC). The mean amount of white blood cells (WBC) was 6.4×10^9/L (± 0.38 SEM) and mean amount of lymphocytes was 1.45×10^9/L (± 0.13 SEM).

Table 1 Baseline characteristics

Characteristic	Study group (*N* = 25)
Sex—no. (%)	
Male	15 (60)
Female	10 (40)
Median age—year (range)	66 (48 – 78)
ECOG performance status—no. (%)	
0	11 (44)
1	11 (44)
2	1 (14)
Unknown	2 (8)
IMDC risk group—no. (%)	
Favorable	5 (20)
Intermediate	15(60)
Poor	4 (16)
Unknown	1 (4)
Median time from initial diagnosis to metastastic disease—mo. (range)	12.5 (0 – 174.5)
Median time from metastastic disease to start study treatment—mo. (range)	20 (1 – 54.5)
Site of metastases—no. (%)	
Lung	18 (72)
Lymph nodes	19 (76)
Bone	6 (24)

Characteristic	Study group (*N* = 25)
Kidney	4 (16)
Other*	8 (32)
Number of metastatic sites—no. (%)	
1	5 (20)
2	9 (36)
3	5 (20)
≥4	5 (20)
Unknown	1 (4)
Previous systemic cancer therapy—no. (%)	
Sunitinib	13 (52)
Pazopanib	8 (32)
Interferon + bevazucimab	1 (4)
Sorafenib	1 (4)
Previous antiangiogenic regimens—no. (%)	
0 or unknown	6 (24)
1	15 (60)
>1	4 (16)

**Adrenal, liver, soft tissue, subcutaneous, peritoneum, breast*

Treatment efficacy and safety

The median time of treatment of patients was 4.2 months (range 0.5–11 months). Two patients (8%) still received treatment at the time of study termination, and all other patients had discontinued study medication due to progression (*n* = 19, 76%) or unacceptable toxicity (*n* = 4, 16%). Median follow-up was 7.9 months (range 0.5–21 months), based on time until death (*n* = 13, 52%) or until time of analysis (*n* = 12, 48%).

At the predefined interim analysis, it became evident that the primary objective of the study, an increase of PFS at 4 months from 50 to 70%, could not be reached. At 4 months, 48% (*n* = 12) of 25 patients had progressive disease. mPFS and mOS were 4.5 months (range 0.5–21 months) and 16 months (range 0.5–20 months), respectively (Fig. 1a, b). Three patients did not show signs of progression at the time of analysis (range 10–21 months) and 11 patients were still alive at the end of the follow-up period (range 10–21 months). The best clinical outcome was stable disease (SD) in 72% (*n* = 18) of the cases. Progressive disease (PD) was observed in 28% (*n* = 7) of the patients. No partial or complete responses were observed.

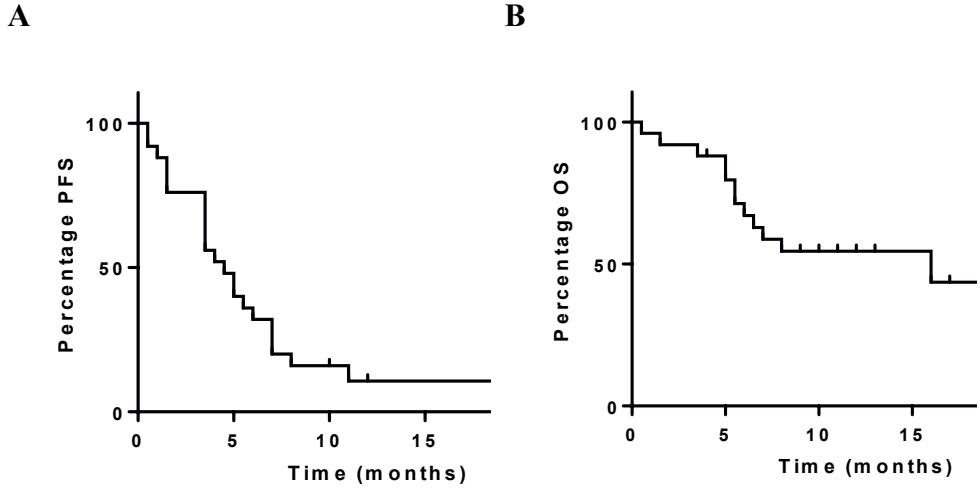


Figure 1. Percentage Progression-free survival and overall survival on treatment. **a** Median PFS is 4.5 months (range 0.5–21 months). At 4 months, 48% (*n* = 12) of 25 patients had progressive disease. **b** Median OS is 16 months (range 0.5–20 months). OS data are preliminary, as 11 patients (44%) were still alive at the end of the follow-up period (range 4–20 months). Data were analyzed using a Kaplan–Meier curve.

Overall, combination treatment was reasonably well tolerated. A total of 168 different AEs was reported, an average of 6.7 per patient. No grade 4 or 5 toxicities were observed. The most common (> 30%) treatment-related toxicities were fatigue (*n* = 11; 44%), anemia (*n* = 10; 40%), pneumonitis (*n* = 10; 40%), anorexia (*n* = 8; 32%) and hypercholesterolemia (*n* = 8; 32%) (Table 2). A total of 18 treatment-related grade 3 AEs were reported in 13 (52%) patients. Grade 3 toxicities included fatigue, anemia, pneumonitis and leukocytopenia. Three patients (12%) endured a DLT related to study medication within 28 days after the start of treatment, i.e., hematuria grade 3, nausea grade 3 and mucositis grade 3. In the case of the patient with hematuria, this resolved upon discontinuation of cyclophosphamide and the patient continued with everolimus treatment until disease progression. For the patient with nausea, study medication was interrupted and, due to rapid disease progression, not reintroduced. In the patient with mucositis, this resolved after a 14 day interruption of study medication and did not recur upon reintroduction of study combination therapy.

Table 2 Treatment-related toxicities, reported in > 10% of patients

Event	Any grade *N* (%)	Grade 1 *N* (%)	Grade 2 *N* (%)	Grade 3 *N* (%)
Constitutional				
Fatigue	11 (44)	3 (12)	5 (20)	2 (8)
Anorexia	8 (32)	2 (8)	6 (24)	
Malaise	6 (24)	2 (8)	2 (8)	2 (8)
Pain	4 (16)	2 (8)	2 (8)	
Fever/chills/flu	3 (12)	3 (12)		
Sweating/flushes	3 (12)	3 (12)		
Dermatology				
Rash	6 (24)	4 (16)	2 (8)	
Pruritus	3 (12)	2 (8)	1 (4)	
Gastrointestinal				
Nausea	7 (28)	4 (16)	2 (8)	1 (4)
Mucositis	7 (28)	4 (16)	2 (8)	1 (4)
Stomatitis	6 (24)	4 (16)	1 (4)	1 (4)
Diarrhea	5 (20)	5 (20)		
Constipation	3 (12)	1 (4)	2 (8)	
Dysgeusia	3 (12)	2 (8)	1 (4)	
Laboratory				
Anemia	10 (40)	1 (4)	7 (28)	2 (8)
Hypercholesteremia	8 (32)	2 (8)	6 (24)	
Hyperglycaemia	6 (24)	1 (4)	4 (16)	1 (4)
Leukocytopenia	6 (24)	1 (4)	3 (12)	2 (8)

Event	**Any grade** ***N*** **(%)**	**Grade 1** ***N*** **(%)**	**Grade 2** ***N*** **(%)**	**Grade 3** ***N*** **(%)**
Hypertriglyceridemia	5 (20)	2 (8)	2 (8)	1 (4)
Thrombocytopenia	5 (20)	4 (16)		1 (4)
Electrolyte disturbance*	4 (16)	4 (16)		
Respiratory				
Pneumonitis	10 (40)	3 (12)	5 (20)	2 (8)
Dyspnea	7 (28)	5 (20)	2 (8)	
Cough	6 (24)	5 (20)	1 (4)	

**Hyponatremia, hypokalemia, hypercalcemia*

Immune monitoring

Based on the immunomonitoring results of the previously performed phase 1 study, a selective panel of immune cell subsets and ratios between immune cell subsets were analyzed in this phase 2 study: total $CD3^+$ T cells, $CD3^+CD4^+$ conventional T-helper cells (Tconv), $CD3^+CD8^+$ CTL, Tregs, effector–suppressor T cell ratio or CD8:Treg ratio, defined as the ratio between $CD8^+$ effector T cells and suppressive Tregs), immunoregulatory and cytotoxic NK cells, cDC1, cDC2 and pDC [31, 37, 38]. In the present study, the total amount of PBMC was 2.01×10^{9}/L ± 0.13 (mean ± standard error of mean (SEM) at baseline and 1.86×10^{9}/L ± 0.14 after 4 weeks of treatment (not significant, NS). The total lymphocyte count was 1.45×10^{9}/L ± 0.13 at baseline and 1.25×10^{9}/L ± 0.11 after 4 weeks of treatment (NS).

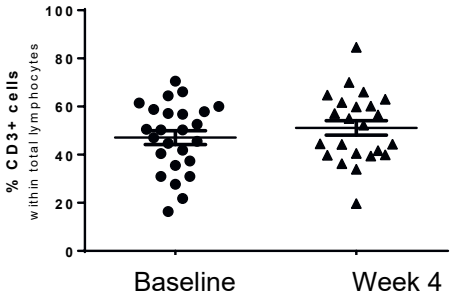
T cell subsets

Neither the frequency nor absolute numbers (AN) of $CD3^+$ T cells in the total lymphocyte population changed significantly during the first 4 weeks of treatment (Fig. 2a). Also, the frequency as well as the absolute numbers of circulating $CD4^+$ T cells did not change significantly (Fig. 2b). Of interest, a small but statistically significant increase in $CD8^+$ CTL was observed in frequency, and a similar, but not significant, trend was seen in absolute numbers (Fig. 2c).

The frequency and absolute numbers of circulating regulatory T cells ($CD4^+CD25^{hi}FoxP3^{hi}$) was found to significantly decrease from baseline to week 4 (Fig. 2d), confirming our previous observations. Of note, although the frequency of circulating Tregs decreased during the first 4 weeks of treatment, expression of the proliferation marker Ki-67 and the inhibitory CTLA-4 receptor in Tregs significantly increased (Fig. 2e, 2f). As the ratio between $CD8^+$ effector T cells and suppressive Tregs (E:S ratio) can have a prognostic impact [40], changes in this ratio were also assessed. As illustrated in Fig. 2g, the E:S ratio significantly increased from baseline to week 4, reflective of a change in the relative distribution between T cell subsets toward a more favorable balance when considering antitumor immune responses.

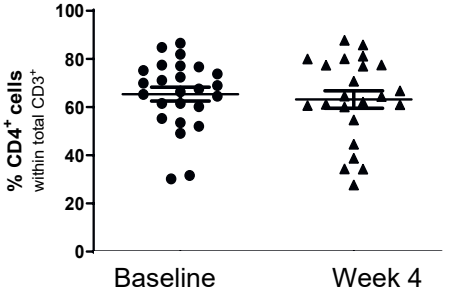
Figure 2.

A



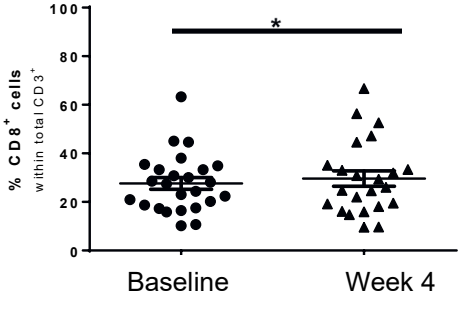
	0 (mean ± SEM)	4 (mean ± SEM)	p
%	47,1%± 2,9	51,1%± 3,0	0,23
AN	0,70 x10^9/L± 0,08	0,65 x10^9/L± 0,06	0,12

B



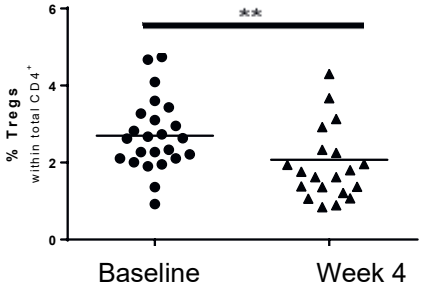
	0 (mean ± SEM)	4 (mean ± SEM)	p
%	65,5%± 2,9	63,9%± 3,7	0,19
AN	0,45 x10^9/L± 0,06	0,37 x10^9/L± 0,04	0,08

C



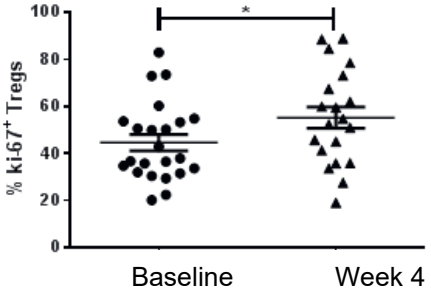
	0 (mean ± SEM)	4 (mean ± SEM)	p
%	27,6%± 2,4	29,7%± 3,2	0,048
AN	0,20 x10^9/L± 0,03	0,23 x10^9/L± 0,04	0,62

D



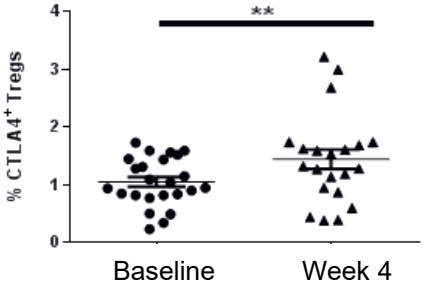
	0 (mean ± SEM)	4 (mean ± SEM)	p
%	2,70% ± 0,19	2,07% ± 0,25	0,002
AN	0,012 x10^9/L± 0,002	0,008 x10^9/L± 0,001	0,012

E



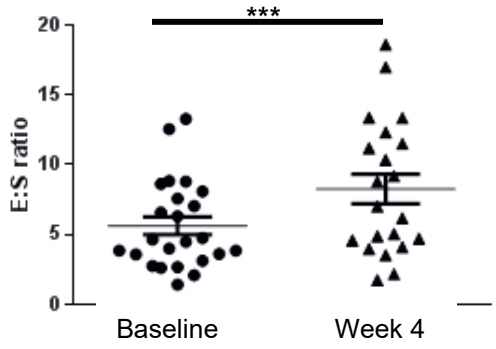
	0 (mean ± SEM)	4 (mean ± SEM)	p
%	44,7% ± 3,5	55,3% ± 4,6	0,016

F



	0 (mean ± SEM)	4 (mean ± SEM)	p
%	1,1% ± 0,1	1,4% ± 0,2	0,008

G



	0 (mean ± SEM)	4 (mean ± SEM)	p
ratio	5,6 ± 0,7	8,3 ± 1,1	0,0004

Figure. 2 Change in lymphocyte subsets between baseline and 4 weeks of treatment. **a** Percentage of T cells ($CD3^+$) in total lymphocytes. **b** Percentage of T helper cells ($CD4^+$) in total $CD3^+$ cells. **c** Percentage of cytotoxic T cells ($CD8^+$) in total $CD3^+$ cells. **d** Percentage of regulatory T cells ($CD4^+CD25^{hi}FoxP3^{hi}$) in total $CD4^+$ cells. **e** Percentage of Ki-67^+ (Ki-$67^+CD4^+CD25^{hi}FoxP3^{hi}$) in regulatory T cells. **f** Percentage of CTLA4$^+$ (CTLA4$^+CD4^+CD25^{hi}FoxP3^{hi}$) in regulatory T cells. **g** E:S ratio. Effector ($CD8^+$):suppressor ($CD4^+CD25^{hi}FoxP3^{hi}$) ratio. Data were analyzed using paired *t* tests. $^*p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Changes in natural killer (NK) cell populations

After 4 weeks of treatment, a shift within the NK cell population occurred. There was a significant decline in both the frequency and absolute number of cytotoxic ($CD56^{dim}CD16^+$) NK cells (Fig. 3a). In contrast, the immunoregulatory ($CD56^{bright}CD16^-$) NK cell population significantly increased in frequency, though not in absolute numbers (Fig. 3b). Overall, the effect of combination treatment of cyclophosphamide and everolimus on the NK cell balance was opposite to the effect observed with T cells and resulted in a more immunoregulatory NK cell profile.

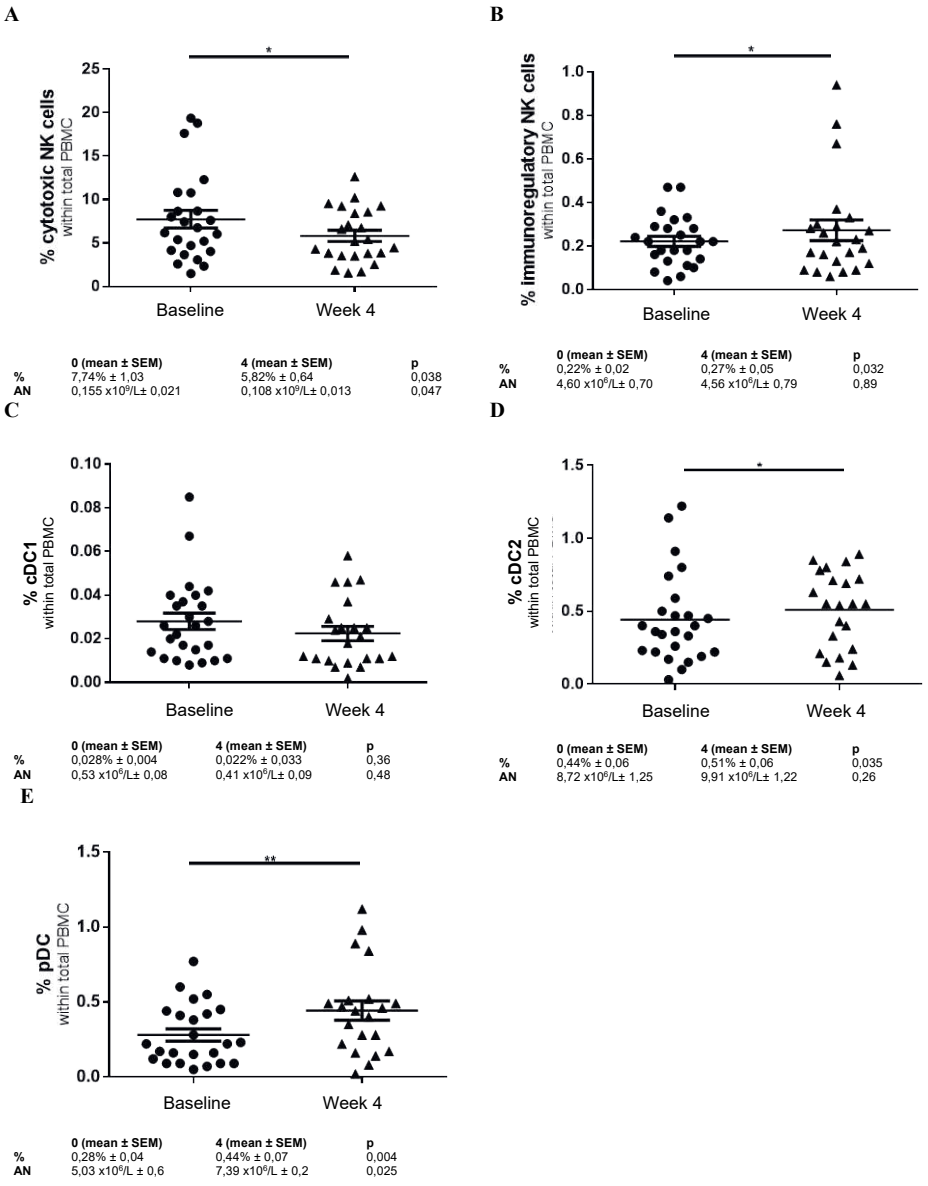


Figure. 3. Change in NK and DC cell populations between baseline and 4 weeks of treatment. **a** Percentage of cytotoxic NK cells ($CD56^{dim}CD16^{+}$) in PBMC. **b** Percentage of immunoregulatory NK cells ($CD56^{bright}CD16^{-}$) in PBMC. **c**. Percentage of cDC1 ($BDCA3^{+}CD14^{-}CD11c^{+}$) in PBMC. **d** Percentage of cDC2 ($BDCA1^{+}CD19^{-}CD14^{-}$ $CD11c^{+}$) in PBMC. **e** Percentage of pDC ($CD11c^{-}BDCA2^{+}CD123^{+}$) in PBMC. Data were analyzed using paired *t* tests. $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Circulating dendritic cell subsets

Several blood dendritic cell subsets were monitored, including myeloid dendritic cells (cDC1 and cDC2) and plasmacytoid dendritic cells (pDC). After 4 weeks of treatment, a small, but non-significant, decrease in cDC1 cells was observed both in frequency and in absolute numbers (Fig. 3c). A significant increase in the frequency, but not in absolute numbers of cDC2 was observed (Fig. 3d). For pDC an increase was demonstrated in frequency as well as in absolute numbers (Fig. 3e). In addition to the frequency of circulating DC subsets, their expression of DC activation markers was monitored (data not shown). The activation status of cDC1, cDC2 and pDC did not significantly change, as measured by the expression of CD40, CD86 and CD123 (the latter only for pDC, data not shown).

Immunomonitoring and correlation with clinical outcome

Overall, combination therapy with low-dose oral cyclophosphamide and everolimus did not improve the clinical outcome of patients when compared to everolimus monotherapy. However, as the combination of cyclophosphamide and everolimus resulted in a significant decrease in Tregs and an increase in the E:S ratio, we explored whether changes in these parameters could be related to the outcome. For this purpose, possible correlations between survival (both PFS and OS) and the percentage of Tregs at baseline, the E:S ratio at baseline, the percentage of Tregs at week 4, the E:S ratio at week 4, the percentual change of Tregs from baseline to week 4 and the percentual change in E:S ratio between baseline and week 4 were assessed (Fig. 4a–d, not all correlations shown). Altogether, a correlation between PFS or OS and either the frequency of Tregs, the E:S ratio or changes herein could not be demonstrated. However, it is noteworthy that in the three patients with the longest PFS (i.e., >1 year), both a decrease in the percentage of Tregs and an increase in the E:S ratio between baseline and week 4 was observed.

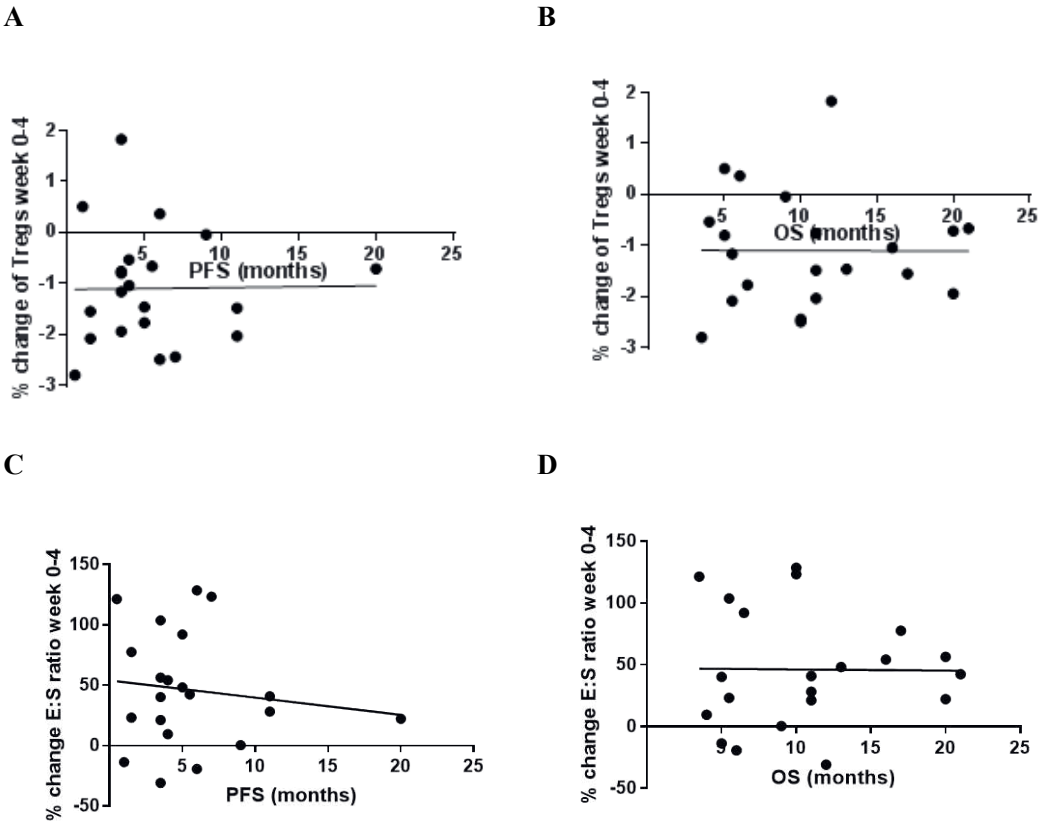


Figure 4. Correlation between survival and changes in Treg frequency and E:S ratio between baseline and 4 weeks of treatment. **a**. Correlation between PFS (months) and relative percentual change in the percentage of Tregs between baseline and 4 weeks of treatment, Pearson $r = 0.014$ ($p = 0.95$). **b** Correlation between OS (months) and relative percentual change in the percentage of Tregs between baseline and 4 weeks of treatment, Pearson $r = -0.004$ ($p = 0.99$). **c** Correlation between PFS (months) and relative percentual change of E:S ratio between baseline and 4 weeks of treatment, Pearson $r = -0.133$ ($p = 0.564$). **d** Correlation between OS (months) and relative percentual change of E:S ratio between baseline and 4 weeks of treatment, Pearson $r = -0.011$ ($p = 0.963$). Data were analyzed using Pearson correlation coefficient. Relative percentual change is the percentage of week 4 minus the percentage at baseline, divided by the percentage at baseline

Discussion

Overall, the results from the present phase 2 study demonstrate that, while the addition of low-dose oral cyclophosphamide to everolimus treatment in patients with clear cell mRCC effectively prevents the everolimus-induced increase in immunosuppressive Tregs, this does not result in clinical benefit. As the predefined goal of the study of improving the PFS rate at 4 months from 50 to 70% was not reached, the study was terminated at the preplanned interim analysis.

Several studies have aimed to lower the amount of Tregs in cancer patients by the administration of cyclophosphamide, with varying results [32, 33, 41, 42]. As there is controversy on the optimal dose and schedule of cyclophosphamide when aiming for Treg depletion and no such data are available for the combination of cyclophosphamide and everolimus, we first performed a phase 1 study in which we set out to determine the optimal Treg-depleting dose of cyclophosphamide when combined with the standard dose of everolimus [37, 38]. In our phase 1 study, continuous once daily oral dosing of 50 mg of cyclophosphamide proved to be most effective in lowering the percentage of Tregs, and therefore this dose was selected for the present phase 2 study. Of note, while we confirmed that once daily oral administration of 50 mg of cyclophosphamide in this phase 2 trial resulted in a reduction in circulating Treg levels after 4 weeks of treatment, an increase in expression of the proliferation marker Ki-67 was observed in Tregs and this was accompanied by an upregulation of the expression of the inhibitory CTLA-4^{+} molecule on Tregs. In accordance with these increased Ki-67 levels, a small rebound in Treg levels was observed after 8 weeks of treatment in the phase 1 part of our study [37, 38]; in the phase 2 part of the study these measurements were not done after 8 weeks. Our observations are in line with results of a study by Ge et al., demonstrating a similar rebound in circulating Treg levels after an initial decrease during the first 14 days of treatment with 50 mg cyclophosphamide once daily in breast cancer patients. This was accompanied by an increase in the proliferative activity of Tregs with a maintained suppressive capacity [33]. Of note, whereas Ge et al. reported a correlation between the temporary reduction in Treg levels and improved clinical outcome, our study showed no relation between a reduction in Tregs and the outcome.

Clearly, the clinical impact of cyclophosphamide-induced effects on Tregs may not only differ per selected cyclophosphamide treatment schedule, but also per tumor type as well as any concomitant treatment such as everolimus in our study. Various mechanisms have been implicated as causative factors for the Treg depletion that is observed, such as the mechanisms mentioned earlier, but also low expression of aldehyde dehydrogenase 1 (ALDH1), inhibition of indoleamine 2,3-dioxygenase (IDO), ATP depletion, CCR2 expression and effects on MDSC; however, it is unknown why the effect of cyclophosphamide on Tregs appears to be temporary [43,44,45].

In our study, the combination of everolimus and cyclophosphamide did not affect the frequency of circulating $CD4^+$ T cells and actually resulted in an increased frequency of $CD8^+$ T cells with a concomitant increase in the E:S ratio. Though the E:S ratio has previously been reported to be significantly associated with improved survival in cancer patients, we did not find a correlation between E:S ratio and survival [40]. Of note, the association between E:S ratio and survival was mostly reported in studies performing analyses in (peri)tumoral tissues instead of peripheral blood [40, 46, 47]. In our study, no serial tumor biopsies were performed precluding similar analyses.

Overall, the balance between the monitored immune cell subsets in our study appeared to shift toward a more robust antitumor immune profile, as illustrated by the selective reduction in the percentage of Tregs and increase in effector $CD8^+$ CTLs as well as blood DC subsets. This did, however, not translate into an enhanced clinical efficacy of combination treatment with everolimus and cyclophosphamide, which may reside in induced changes in the NK cell population, as an increase in the $CD56^{bright}$ immunoregulatory NK cell population and a decrease in the $CD56^{dim}$ cytotoxic NK cell population were observed. The change in the balance between both NK cell populations can be attributed to cyclophosphamide, as an opposite effect (i.e., a decrease in immunoregulatory NK cells and an increase in cytotoxic NK cells) was observed in the phase 1 patients treated with everolimus monotherapy [31]. A possible explanation might be the preferential apoptosis of $CD56^{dim}$ cytotoxic NK cells, as postulated by Bauernhofer et al. [48].

It will be interesting to explore whether therapeutic approaches that can counteract this putative detrimental effect of cyclophosphamide on the NK cell population can improve clinical antitumor activity of the combination of everolimus and cyclophosphamide.

For example, the TKI axitinib and the anti-epileptic drug valproic acid have been reported to in vitro increase expression of NKG2D ligands on tumor cells, thereby increasing their susceptibility to NK cell and γδ T cell recognition [49, 50]. Alternatively, very low doses of recombinant IL-2 and IFN-α could be considered, as these were reported to increase NK cell numbers in vivo, albeit that these consisted mainly of the $CD56^{bright}$ cell subset and they will probable increase Treg numbers as well [51]. Potential drawbacks for such triple combination treatment regimens are related to an increased risk of toxicity. For example, studies combining a VEGF TKI with an mTOR inhibitor in mRCC were mostly terminated prematurely as a result of significant toxicity [52, 53].

As stated before, among others, the PD-1 checkpoint inhibitor nivolumab has replaced everolimus as the standard second-line therapy in mRCC patients. Future studies investigating whether nivolumab can efficiently counteract the immunosuppressive effects observed with everolimus monotherapy may be considered and could potentially result in more potent antitumor activity than either treatment alone.

In conclusion, results from the present phase 2 clinical study demonstrate that addition of low-dose metronomic cyclophosphamide to everolimus can effectively prevent the everolimus-induced increase in Tregs in mRCC patients and in addition results in an increased frequency of $CD8^+$ CTL, cDC2 and pDC. The Treg-depleting effect diminished over time (as demonstrated in the phase 1 study [37, 38]), which may be related to the observed increase in Ki-67^+ levels in Tregs and was accompanied by a minor increase in Treg $CTLA4^+$ expression, a decline of cytotoxic NK cells and an increase of immunoregulatory NK cells. Overall, the immunomodulatory effects of the combination of metronomic cyclophosphamide and everolimus did not translate into an altered clinical outcome as measured by the percentage of patients progression free after 4 months of therapy. The comprehensive immunomonitoring analysis performed in this study provides relevant insight for the rational design of future therapeutic approaches in mRCC and other malignancies such as neuroendocrine tumors, in which mTOR inhibitors are also used as anti-cancer therapeutics.

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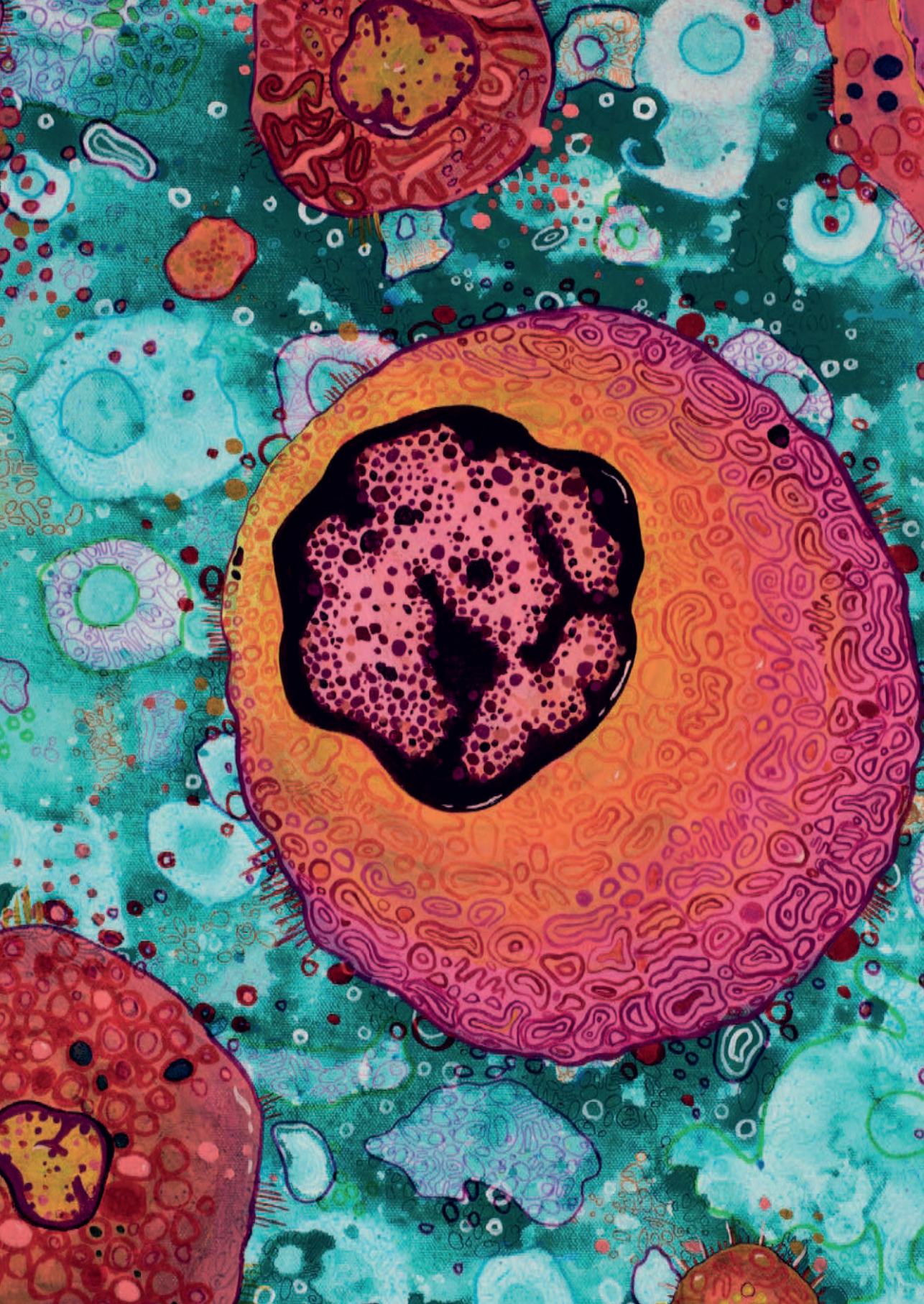
Part Two: Effect of granulocyte (monocyte) colony stimulating factor and Her2 targeting in breast cancer patients



Chapter 4 Immune modulation and long-term survival of patients with locally advanced breast cancer on GM-CSF-supported neoadjuvant chemotherapy (the Spinoza-trial)

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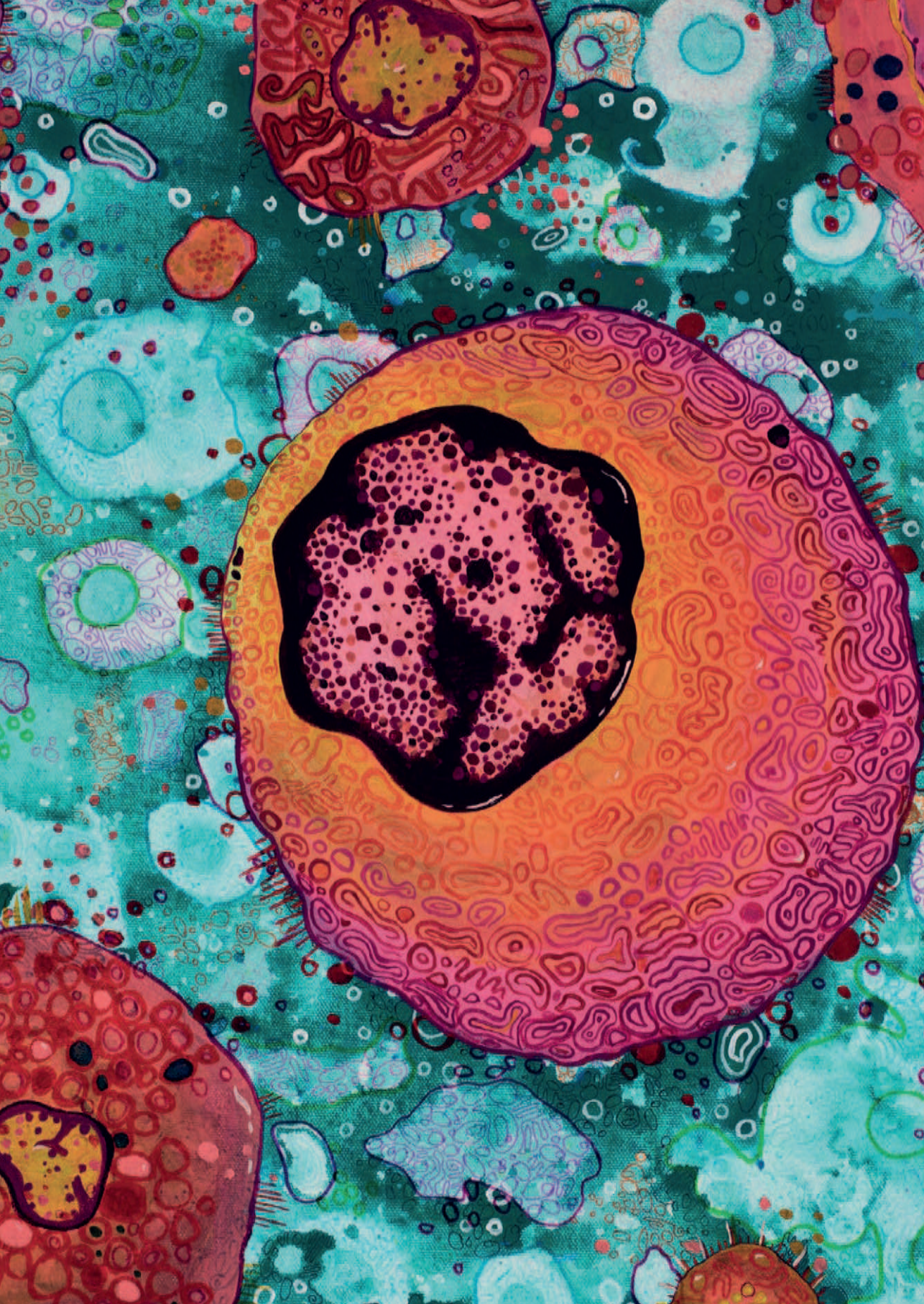


Abstract

Background: Neoadjuvant chemotherapy (NAC) is standard-of-care for treatment of high-risk early-stage breast cancer (BC). When combined with immune stimulation it may also act as an in vivo anti-tumor immunization regimen. In this context, balance between immunostimulatory dendritic cell (DC) subsets on the one hand, and regulatory T cells (Tregs) and myeloid regulatory cells on the other hand, may be decisive in ultimate clinical outcome.

Methods: We therefore studied the effects of 6 cycles of myeloid growth factor-supported NAC (with high doses of doxorubicin and cyclophosphamide) on immune effector subsets of patients with locally advanced BC (LABC), who participated in the Phase-III Spinoza trial (NTR170/NL136) between 1999 and 2002. Patients were randomized for systemic administration of either GM-CSF (n=14), known for its DC-stimulatory effects, or G-CSF, which is commonly used for haematopoietic support (n=20).

Results: The GM-CSF treated patients had a non-significant improved DFS and OS in comparison to G-CSF-treated patients (10-year DFS 64.2% versus 45% and 10-year OS 64.2% versus 50%), survival based on stage difference, tumor type or receptor status did not differ significantly. In general, systemic frequencies of both DC and monocyte subsets increased over treatment with either growth factor. Flowcytometric analysis of the tumor-draining lymph nodes (TDLN) revealed increased numbers of migratory mature DC in GM-CSF rather than G-CSF administered patients ($p = 0,018$).

Conclusion: Our data revealed enhanced systemic DC differentiation upon NAC supported by GM-/G-CSF. GM-CSF had additional stimulatory effects on migratory DC in TDLN that might explain the observed trend for increased clinical benefit.

Introduction

Breast cancer (BC) is the third most common cancer overall (1.9 million cases) and is the leading cause of cancer death in females worldwide (181,004 deaths) (1). Around a third of women present with advanced disease stage III and IV. For regional BC 5-year survival is 86%, but drops to 29% when distant metastases are present (2).
Locally advanced BC (LABC) represents a heterogeneous group of breast malignancies with different biological and clinical characteristics. LABC includes stage IIB (tumor > 5 cm), IIIA and IIIB BC according to the American Joint Committee on Cancer (AJCC) (3). The management and diagnosis of LABC has evolved over the years. With local therapy alone distant metastases usually appear rapidly, indicating that most of these patients already have micrometastases at the time of diagnosis. Neoadjuvant chemotherapy (NAC) treatment has in the past decade become common practice in high-risk early-stage BC; it can induce a tumor response before surgery, leading to down-staging, and thereby improve cosmetic outcomes after surgery and/or limit the extent of axillary lymph node removal, as well as eliminating (distant) micrometastases. Moreover, pathological complete response (pCR) upon NAC is associated with improved outcome, especially for triple negative (TN) and Her2+ BC, thus providing patients and treating physicians with prognostic information (4). NAC can improve disease-free survival (DFS) and overall survival (OS) in LABC, and has become standard of care for this disease stage (5).
There has been a resurgence of interest in combined chemo- and immunotherapy for the treatment of cancer. Cytostatic drugs can induce so-called immunogenic cell death, marked by the simultaneous release of tumor-derived antigens and damage-associated molecular patterns (DAMPs) that can induce activation of dendritic cells (DC) and mediate subsequent cross-priming of cytotoxic T lymphocytes (CTLs) (6). Indeed, anthracyclin efficacy in a murine BC model was shown to depend on Toll-Like Receptor (TLR)-3-mediated induction of a type-I interferon (IFN) response and the expression of CXCL10, a chemokine mediating the attraction of effector CTLs to the tumor (7). This has led some to postulate that chemotherapy may in fact be a form of immunotherapy, which is supported by the finding that immune parameters of the host immune response can serve as a molecular signature for chemotherapy response prediction (8, 9).

In patients with BC, especially TN and Her2+, undergoing NAC, tumor-infiltrating T cells were found to be predictive for achieving pCR (10-12). Moreover, chemotherapy-induced tumor cell killing may contribute to a reduction in tumor-derived immunosuppressive factors that contribute to myeloid dysfunction and tumor immune escape, as shown by for example IL-6 and NK-cell responses (13, 14). Chemotherapy was shown to normalize myeloid profiles in both tumor-bearing mice and patients, improving antigen presentation capacity and facilitating the induction of an anticancer immune response (15). T cell infiltration was also reported to predict for responsiveness of especially TNBC to immune checkpoint blockade (16). In early-stage TNBC combined NAC and immune checkpoint blockade has shown promising increases in pCR rates and event-free survival (reviewed in (17)) leading to the approval of neoadjuvant pembrolizumab with NAC for patients with early-stage TNBC (18).

While now a major focus of clinical research, over two decades ago the concept of combined neoadjuvant chemo- and immunotherapy was not widely supported and checkpoint inhibitors were not available yet in the clinical setting. At that time we nevertheless postulated that this approach would make optimal use of the primary tumor as a source of antigens resulting in CTL priming in the tumor-draining lymph nodes (TDLN) (19-21). To test this concept a randomized Phase-III trial was initiated to compare the efficacy and immune modulating effects of NAC (6 neoadjuvant chemotherapy cycles or split-course 3 neoadjuvant and 3 adjuvant cycles) combined with GM- versus G-CSF (the Spinoza trial, start inclusion 1999). In the Spinoza trial high doses of doxorubicin and cyclophosphamide were administered pre-operatively. Patients were also treated with bone marrow stimulation factor (mandatory). It was hypothesized that combining NAC with GM-CSF, a known DC mobilizing and activating factor, would overcome BC-associated immune suppression and optimize the uptake and presentation of released tumor antigens by DCs and thus facilitate effective priming of antitumor immunity. In this report we have therefore assessed the systemic and local effects of GM-CSF versus G-CSF on both immunostimulatory and immunosuppressive subsets in a subgroup of patients with LABC who were enrolled in the Spinoza trial in the Netherlands. Moreover, we determined the prognostic value of the rates of these immune cell subsets in peripheral blood and TDLN.

Patients and Healthy Controls

The Phase-III Spinoza trial (NTR170/NL136) enrolled 77 patients with LABC from February 1999 to December 2002 across 6 medical centers in 3 different countries, instead of the planned accrual of 720 patients. This was the result of a sudden discontinuation of the GM-CSF molgramostim (Leucomax®) by Novartis. In two Dutch centers, VU University Medical Center (VUmc) and Amstelveen Medical Center Amstelland, 34 of these 77 patients were included. For survival analysis, data from these 34 patients were available (Table I for patient characteristics). The accrual in the immune monitoring side-study described here was limited to 16 patients (Supplemental Table I for patient characteristics). TDLN of 12 patients from the immune-monitoring side study were available (Supplementary Figure 1 Inclusion of patients). Eighteen age-matched healthy women (mean age 48.8 years) gave informed consent to be included in the immune monitoring side study. For survival analysis, data from 34 patients treated in these same two medical centers were available (see Table I for patient characteristics). The study and immune monitoring side-study were approved by the Medical Ethical Committee of the VUmc. Patients were enrolled after written informed consent was obtained. Patients were randomized between either 6 courses of neoadjuvant doxorubicin (90 mg/m2) and cyclophosphamide (1000 mg/m2) by intravenous bolus injection or a split-course administration of 3 neoadjuvant and 3 adjuvant cycles. Cycles were repeated every 21 days. Based on a dose-finding study, a scheduled dose-reduction of 10% of the previous dose level was applied in cycles 2 and 4 (Supplementary Figure 2) (22, 23). A second randomization was between supplemental treatment with either molgastrim GM-CSF (Leukine®) or filgastrim G-CSF (Neupogen® /Granulokine®). GM-CSF 250 μg/m^2 s.c. was daily administered from days 2-12. G-CSF 5 μg/kg/day s.c was similarly administered from day 2-12. Major inclusion criteria were: histologically proven LABC stage IIB with a primary BC larger than 5 cm, IIIA or IIIB according to the AJCC criteria 5th edition, and adequate hematological, renal and hepatic functions. Patients who received prior chemotherapy, radiotherapy, or hormone therapy were excluded. Local tumor response was monitored according to RECIST as clinical partial or complete response (cPR or cCR).

After surgery, pathological partial or complete response was assessed (pPR of pCR in both breast as well as lymph nodes). Hormone receptor (HR)+ was defined as > 10% estrogen and/or progesterone receptor positive cells, determined by immunohistochemistry (IHC). DFS was defined as the time between the day of enrolment and the date of evidence of disease specific recurrence. OS was defined as the time between the day of enrolment and the date of death. For DFS and OS analysis, data collection was closed on January 31st, 2020. Supplemental Figure 1 shows the flowchart of the study design and the blood sampling schedule.

Flowcytometric analyses

Peripheral blood was drawn prior to the first treatment cycle and prior to every subsequent cycle of chemotherapy and from one blood collection of 18 age-matched healthy women. Peripheral blood mononuclear cells (PBMC) were isolated from patients and healthy donors by density gradient centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway) before cryo-preservation. Four-color flow cytometry was performed to determine the number and the activation state of distinct subsets of $CD11c^+$ conventional dendritic cells (cDC) and plasmocytoid DC (pDC) as described previously (24). In addition, monocytes, monocytic myeloid derived suppressor cells (mMDSC) and regulatory T cells (Tregs) were enumerated (24, 25). Samples were analyzed on a FACSCalibur using Cellquest FACS analysis software (BD, Franklin Lakes, NJ). Monoclonal antibodies directed against the following antigens and their corresponding isotype controls were used: BDCA-1, BDCA-2, BDCA-3 (Miltenyi Biotec, Bergisch Gladbach, Germany); CD11c, CD123 (BD Biosciences, San Jose, CA), CD14, CD86, HLA-DR (BD Pharmingen, San Diego, CA), CD40 (Immunotech, Marseille, France). M-DC8 (a generous gift from Dr E. Peter Rieber, University of Munich, Munich, Germany) was used to detect 6-sulfo LacNAc. type-2 cDC (cDC2) were defined as $CD11c^{hi}CD14^-$BDCA-1/$CD1c^+$, cDC1 as $CD11c^+CD14^-$BDCA-3/$CD141^+$, and non-classical monocytes as $CD11c^{hi}CD14^{int}$M-$DC8^+$; additional analysis showed the latter to be $CD16^+$, as described for non-classical monocytes. pDC were identified as $CD11c^-$BDCA-2^+CD123^+. Monocytes were defined as $CD11c^{hi}CD14^{hi}$HLA-DR^+ and mMDSC as HLA-$DR^{lo}CD14^{hi}$, as previously described (26, 27). Data were expressed as percentages of PBMC after exclusion of granulocytes and cell debris, based on forward and sideward light scatter properties.

To assess the maturation status of the PBDC subsets, median fluorescence indices (MFI) of HLA-DR, CD40 and CD86 were calculated by dividing the median fluorescence of the test antibody by the median fluorescence of the isotype-matched control antibody (BD Biosciences, San Jose, CA). T-regulatory cells (Tregs) were defined as $CD3^+$, $CD4^+$ and $CD25^{hi}$, and expressed as percentages of $CD3^+CD4^+$ cells; additional analyses confirmed these as *bona fide* Tregs, based on high expression levels of both FoxP3 and CTLA-4 (25, 28, 29).
We analyzed measurements at baseline (pre-treatment) and after 3 or 6 cycles of treatment. If no sample at cycle 6 was available, measurements after 5 cycles were included in the respective analyses, which showed no deviations from the other cycle-6 data (n=1 for GM-CSF; n=2 for G-CSF).

Analysis of TDLN DC

Single-cell suspensions from TDLN of 12 patients were analyzed for the presence of DC after 6 cycles of chemotherapy with GM-CSF (n = 7) or G-CSF (n = 5). TDLN were excised from surgical mastectomy specimens by the pathologist and bisected. Viable cells were scraped from the cutting surface with a surgical blade, enzymatically dissociated, and analyzed by flow cytometry as previously described (30). Conventional migratory DC were detected by CD1a expression and their activation state assessed by CD83 and CD86 double-staining as described (CD1a and CD86 mAbs, BD Biosciences, San Jose, CA; CD83 mAb Immunotech, Marseille, France) (31).

Statistics

Fisher exact test and paired T-test compared baseline values of patients. Differences between baseline, cycle 3 and post-treatment immune cell frequencies were compared by mixed effect analysis. Patient and healthy donor data were compared employing an unpaired T-test to determine statistically significant differences. Kaplan-Meier plots and Pearson correlation coefficients were applied to determine the significance of differences in DFS and OS. In all statistical analyses, differences were considered to be statistically significant when $P \leq 0{,}05$.

Results

Clinical efficacy and toxicity

There were no significant differences in baseline characteristics from enrolled patients (n=34; Table 1) or immune monitoring side study (n=16; Supplementary Table 1). The mean tumor size was approximately 7cm, in line with the eligibility criteria, selecting patients with LABC. All patients but one received 6 cycles of chemotherapy, underwent mastectomy, and received adjuvant radiotherapy and hormonal therapy when appropriate. One patient received only 4 of the 6 planned chemotherapeutic treatment cycles and refused further chemotherapy and surgery. This patient received radiotherapy and hormonal therapy. Response evaluation according to RECIST was performed in all patients at the end of neoadjuvant therapy. Over 90% of patients achieved a partial or complete clinical response (cPR or cCR) on radiological evaluation during NAC, and 6 patients achieved a pathological complete response (pCR) at surgery (Table 1). Except for one patient experiencing transient grade 4 neutropenia and one patient experiencing transient grade 3 lymphopenia, no or mild hematological toxicity was observed according to the Common Terminology Criteria for Adverse Events (CTC-AE). With regards to patient characteristics no significant differences between treatment arms were observed.

At the time of analysis (follow-up range 18 - 22 years) 14 out of 34 patients were still alive (41%). The 5 year DFS rate was 64.7% and 5 year OS rate was 70.6%. The 10 year DFS rate was 52.9% and 10 year OS rate was 55.9%. Median OS (mOS) was 16.3 years (range 1.5 – 20.9 years) with a pCR rate of 17.6% (Table 1 and Figure 1A). There was a trend, though not statistically significant, for increased 5 and 10 year DFS rate in patients treated with GM-CSF as compared to G-CSF (respectively 78.6% vs 55%; p 0.28, and 64.2% vs 45%; p 0.32). Similarly, 5 and 10 year OS rate for GM-CSF treated patients was non-significantly increased compared to G-CSF, respectively 78.6% compared to 65% (p 0.47) and 64.2% vs 50% (p 0.50) (Table 1 and Figure 1B).

In this group of patients with large tumors at presentation, pCR was achieved in only 6 cases (17.6%). Despite an apparent trend of increased survival benefit, the GM-CSF group achieved less pCR than the G-CSF group (7% vs 25%, p 0.36), and had a lower proportion of hormone sensitive tumors (78% vs 85%, 0.67) (Table 1).

Low patient numbers call for caution in interpretation of the the data. Overall, OS was non-significantly reduced in patients not achieving pCR (pCR vs no pCR: %10 year OS 83.3% vs 51.9%, HR 0.45, 95% CI 0.12-1.70, p 0.33) (Figure 1C). OS for HR+ or HR- and Her2+ or Her2- did not differ significantly (HR+ %10-year OS 57.1% vs 40%, HR 0.66, 95% CI 0.16–2.75, p 0.66 and Her2+ %10-year OS 66.7% vs 30%, HR 0.52, 95% CI 0.10–2.64, p 0.61) (Figure 1D,1E).

Table 1: Patient Characteristics	**All (n)**	**GM-CSF (n)**	**G-CSF (n)**	**p-value GM- vs G-CSF**
Number of patients (n)	34	14	20	
Mean age (years)	50.8	49.9	51.5	0.567[a]
Histology				
-Ductal	79% (27)	71% (10)	85% (17)	0.410[b]
-Lobular	18% (6)	29% (4)	10% (2)	0.202[b]
-Other	3% (1)	0% (0)	5% (1)	>0.999[b]
Receptor status				
-HR+/Her2 unknown	47% (16)	50% (7)	45% (9)	>0.999[b]
-HR+/Her2-	27% (9)	14% (2)	35% (7)	0.250[b]
-HR+/Her2+	9% (3)	14% (2)	5% (1)	0.556[b]
-HR-/Her2 unknown.	12% (4)	7% (1)	15% (3)	0.627[b]
-TN	3% (1)	7% (1)	0% (0)	0.412[b]
-Unknown	3% (1)	7% (1)	0% (0)	0.412[b]
Stage				
-IIb	18% (6)	21% (3)	15% (3)	0.672[b]
-IIIa	50% (17)	57% (8)	45% (9)	0.728[b]
-IIIb	33% (11)	21% (3)	40% (8)	0.295[b]
Mean size BC (mm)	69.9	63	74.8	0.377[a]
Mean number of tumor-positive lymph nodes	3.8	3.9	3.7	0.879[a]
Tumor response to neo-adjuvant therapy (n)	29% (10)	14% (2)	40% (8)	0.141[b]
-cCR	62% (21)	79% (11)	50% (10)	0.153[b]
-cPR	17.6% (6)	7% (1)	25% (5)	0.364[b]
-pCR				
mOS (years)	Not reached	Not reached	9.75	0.339[c]
5 year DFS	64.7% (22)	78.6% (11)	55% (11)	0.275[b]
10 year DFS	52.9% (18)	64.2% (9)	45% (9)	0.315[b]
5 year OS	70.6% (24)	78.6% (11)	65% (13)	0.467[b]
10 year OS	55.9% (19)	64.2% (9)	50% (10)	0.495[b]

[a]unpaired T-test [b]Fisher's exact test [c]Log-Rank Mantel-Cox

Figure 1: Overall survival of entire study population and subgroups

A. Kaplan-Meier OS of LABC-patients

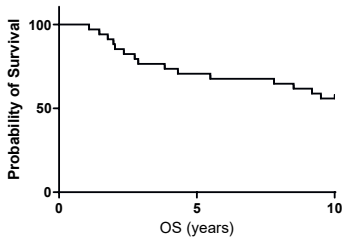
B. Kaplan-Meier OS of LABC-patients treated with either G-CSF or GM-CSF

C. Kaplan-Meier OS of LABC-patients achieving pCR versus no pCR

D. Kaplan-Meier OS of LABC-patients with HR+ versus HR-

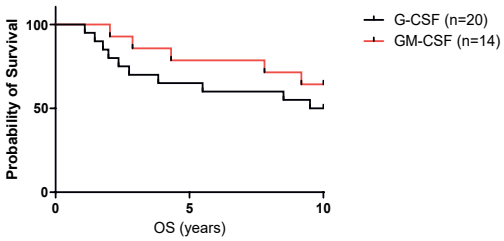
E. Kaplan-Meier OS of LABC-patients with Her2+ versus Her2-

A



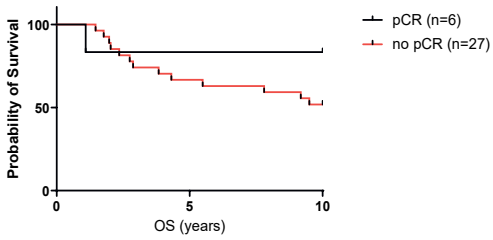
%10-year survival 55.9%

B



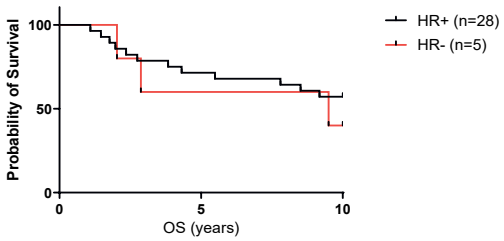
%10-year survival 64.3% vs 50%
mOS GM-CSF not reached vs 9,75 years
HR 1.64, 95% CI 0.59-4.54, p 0.34

C



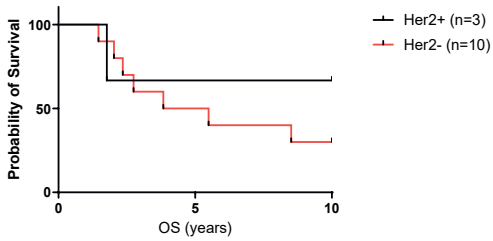
%10-year survival 83.3% vs 51.85%
mOS not yet reached vs not yet reached
HR 0.45, 95% CI 0.12-1.70, p 0.33

D



%10-year survival 57.1% vs 40%
mOS not reached vs 9.5 years
HR 0.66, 95% CI 0.16–2.75, p 0.66

E



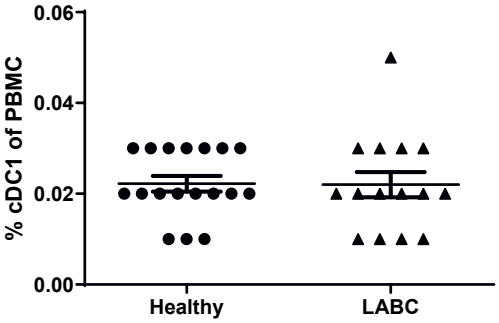
%10-year survival 66.7% vs 30%
mOS not yet reached vs 4.67 years
HR 0.52, 95% CI 0.10–2.64, p 0.61

Peripheral blood myeloid subset analysis in healthy donors versus patients with LABC

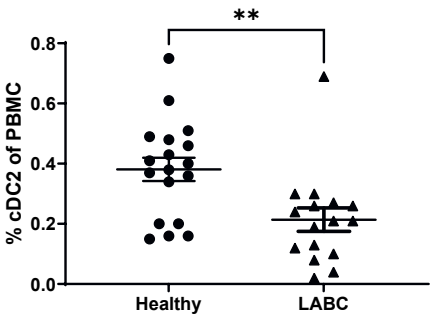
We hypothesized that GM-CSF would have favorable modulatory effects on myeloid cell subsets in terms of balancing immune stimulatory DCs vs suppressive myeloid cells. Therefor, we first compared these cellular subsets in blood specimens from healthy women (mean age 48.8 years) with baseline specimens from the enrolled LABC patients (Figure 2). Significant differences were observed for cDC2, non-classical monocytes, mMDSC and Tregs, with lower frequencies in patients versus healthy controls. Also a non-significant difference in pDC was observed with slightly lower rates in the patients with LABC. The expression levels of the DC activation markers CD40, CD86, and HLA-DR on any of the tested DC subsets were comparable (data not shown). Of note, we did not find a difference in the studied immune parameters between the patients with a split-course of neoadjuvant (3 courses) and adjuvant (3 courses) therapy and the patients with 6 courses of neoadjuvant therapy. For the purpose of this translational analysis of 16 patients, the chemotherapy administration regimens were lumped together regardless if treatment was done with a split-course of 3 neoadjuvant plus 3 adjuvant courses, or 6 neoadjuvant courses of therapy. In this analysis, patients were divided between GM- or G-CSF treatment randomization.

Figure 2: Immune cell subsets healthy vs LABC

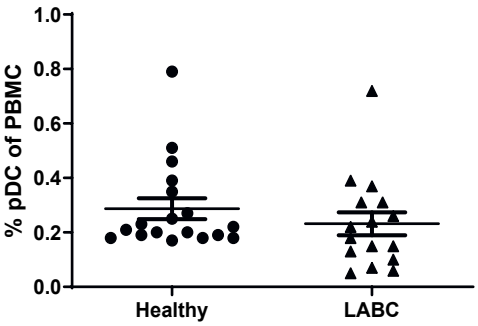
Frequencies of peripheral blood DC, (non-classical) monocytes and mMSDC as percentage of PBMCs. Comparison between healthy women and patients with LABC. *, $P < 0,05$



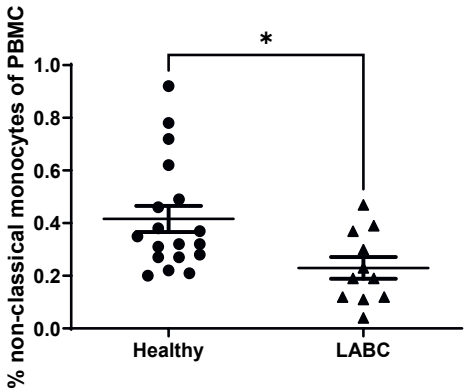
H (mean ± SEM)	LABC (mean ± SEM)	p
0.02%± 0.00	0.02%±0.00	0.945
N=18	N=16	



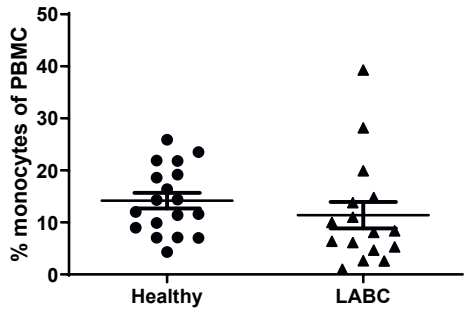
H (mean ± SEM)	LABC (mean ± SEM)	p
0.38%± 0.04	0.22%± 0.04	0.004
N=18	N=17	



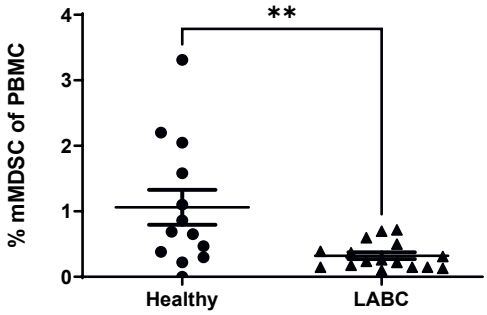
H (mean ± SEM)	LABC (mean ± SEM)	p
0.29%± 0.04	0.23%±0.04	0.337
N=18	N=17	



H (mean ± SEM)	LABC (mean ± SEM)	p
0.42%± 0.05	0.23%± 0.04	0.015
N=18	N=17	



H (mean ± SEM)	LABC (mean ± SEM)	p
14.19%±1.52	11.09%±2.43	0.282
N=18	N=17	



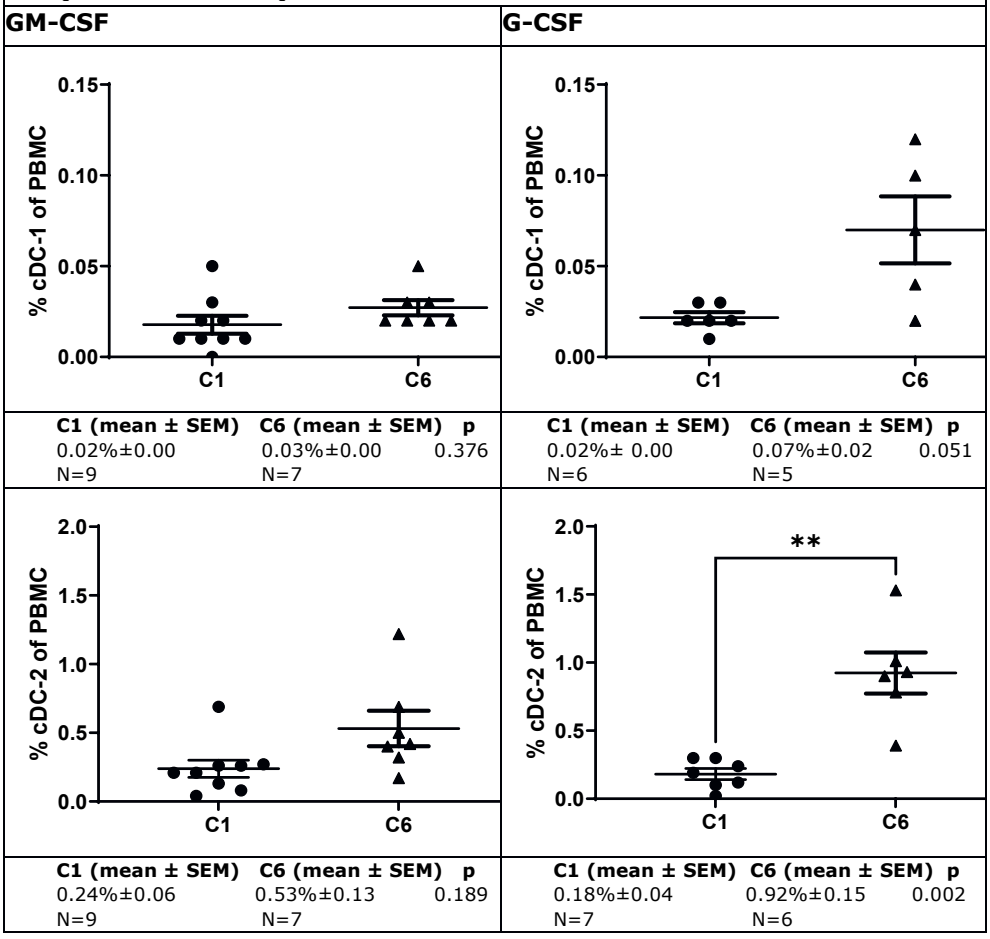
H (mean ± SEM)	LABC (mean ± SEM)	p
1.06%± 0.27	0.32%± 0.05	0.004
N=13	N=16	

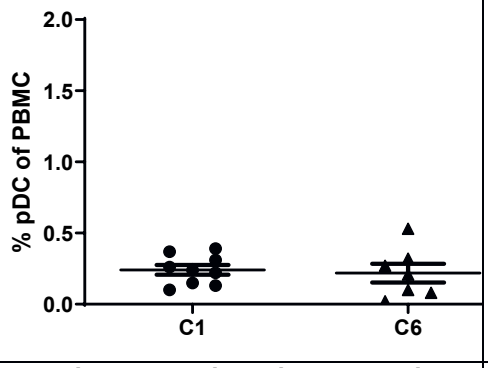
Dynamics of myeloid subset during NAC combined with GM-CSF versus G-CSF

We then evaluated these immune monitoring parameters in patients treated with GM- or G-CSF. The peripheral blood frequencies of most myeloid subsets increased during NAC, both in the GM- and G-CSF arms, as compared between cycle 1 (C1) and cycle 6 (C6) (Figure 3). On the whole, more prominent increases in DC and monocyte subset frequencies were observed on G-CSF treatment, with significance levels reached for cDC2, pDC and non-classical and classical monocytes. None of the myeloid subset rates (either at baseline or changes on treatment) were significantly correlated to DFS or OS (data not shown).

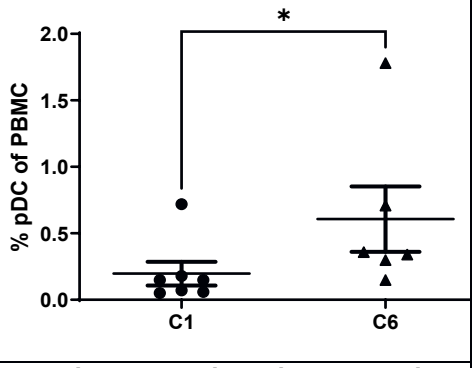
Figure 3: Immune cell subsets GM-CSF vs G-CSF

Frequencies of peripheral blood myeloid cell subsets as percentage of PBMCs. Comparison between patients treated with either GM-CSF or G-CSF. *, $P < 0,05$

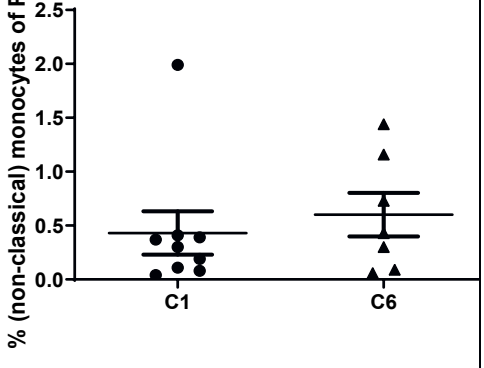




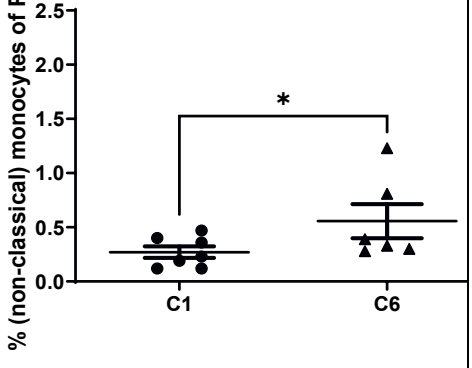
C1 (mean ± SEM)	C6 (mean ± SEM)	p
0.24%±0.03	0.22%±0.07	0.634
N=9	N=7	



C1 (mean ± SEM)	C6 (mean ± SEM)	p
0.20%± 0.09	0.61%±0.25	0.041
N=7	N=6	



C1 (mean ± SEM)	C6 (mean ± SEM)	p
0.43%±0.20	0.60%±0.20	0.721
N=9	N=7	



C1 (mean ± SEM)	C6 (mean ± SEM)	p
0.27%± 0.05	0.56%±0.16	0.049
N=7	N=6	

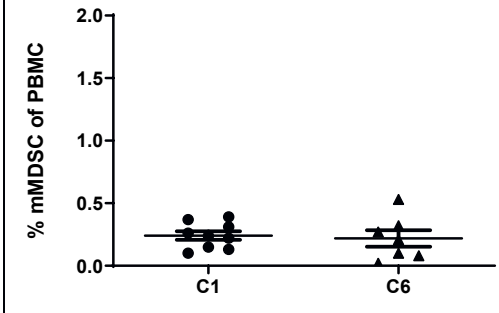
Dynamics of suppressive cell subset during NAC combined with GM-CSF versus G-CSF

Interestingly, mMDSC levels in peripheral blood (non-significantly) increased only on NAC with G-CSF treatment but were not affected by NAC with GM-CSF group (Figure 4A and 4B). As Tregs were previously reported to increase upon systemic GM-CSF administration, we monitored their frequencies in peripheral blood during treatment and found them to be numerically increased in both treatment arms but this finding was not statistically significant (Figure 4C and 4D). MDSC at baseline or changes with treatment did not correlate with either DFS or OS (Figure 4E). Low baseline rates of Tregs significantly correlated with a prolonged OS (Figure 4F). As mentioned before, other immune cell subsets did not correlate with survival.

Figure 4: Immune suppressor cells C1 vs C6 and OS

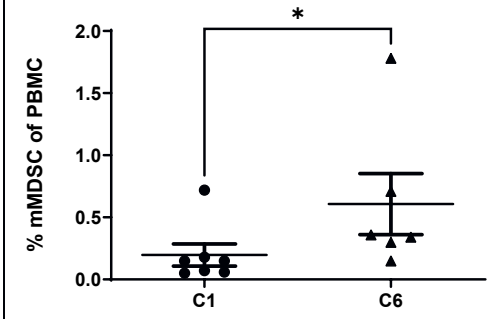
A. Frequencies of peripheral blood mMDSC as percentage of PBMCs. Comparison between C1 and C6 in patients with LABC treated with GM-CSF. *, $P < 0,05$
B. Frequencies of peripheral blood mMDSC as percentage of PBMCs. Comparison between C1 and C6 in patients with LABC treated with G-CSF. *, $P < 0,05$
C. Frequencies of regulatory cells (Tregs, $CD4^+$ $CD25^{hi}$ cells) as percentage of $CD4^+$ T cells. Comparison between C1 and C6 in patients with LABC treated with GM-CSF. *, $P < 0,05$
D. Frequencies of -regulatory cells (Tregs, $CD4^+$ $CD25^{hi}$ cells) as percentage of $CD4^+$ T cells. Comparison between C1 and C6 in patients with LABC treated with G-CSF. *, $P < 0,05$
E. Pearson correlation of baseline values of peripheral mMDSC correlated to OS
F. Pearson correlation of baseline values of peripheral Tregs correlated to OS

GM-CSF

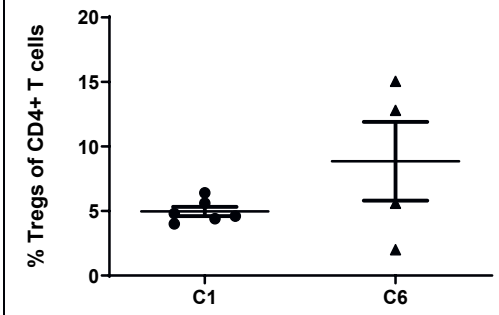


C1 (mean ± SEM)	C6 (mean ± SEM)	p
0.24%± 0.03 N=9	0.22%±0.07 N=7	0.634

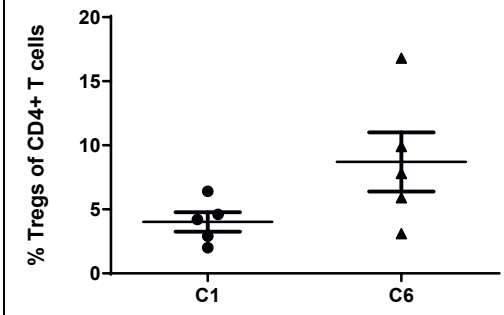
G-CSF



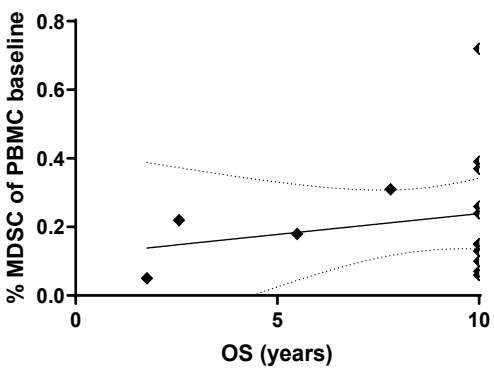
C1 (mean ± SEM)	C6 (mean ± SEM)	p
0.20%± 0.09 N=7	0.61%±0.25 N=6	0.041



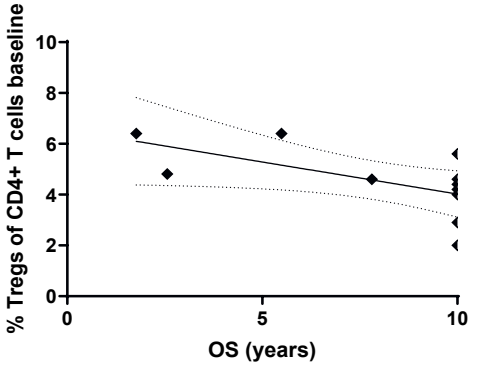
C1 (mean ± SEM)	C6 (mean ± SEM)	p
4.97%± 0.36 N=6	8.86%±3.05 N=4	0.257



C1 (mean ± SEM)	C6 (mean ± SEM)	p
4.02%± 0.75 N=5	8.70%±2.31 N=5	0.057



Pearson r 0.20 (p 0.454), n=16



Pearson r -0.61 (p 0.047), n=11

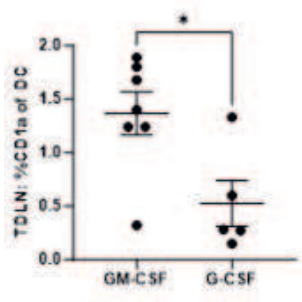
Increased frequencies of mature migratory DCs in TDLN of GM-CSF treated patients

We also studied migratory CD1a$^+$ cDC content and activation state in TDLN, which we previously found to be increased upon local GM-CSF administration in patients with early-stage melanoma (32). In the current study, after 6 treatment cycles, patients treated with GM-CSF had significantly higher CD1a$^+$ cDC frequencies in TDLN as compared to patients treated with G-CSF, 1.38% vs 0.53%, p = 0.018, (Figure 5A). Of note, regardless of previous NAC treatment these cDC uniformly expressed CD83 and CD86, showing them to be mature and capable of T cell activation (Figure 5B) (31). CD83 and CD86 levels on these CD1a$^+$ DC demonstrated a further enhanced maturation state upon GM-CSF treatment as compared to G-CSF, albeit not significantly in these small patient numbers (Figure 5B).

Figure 5

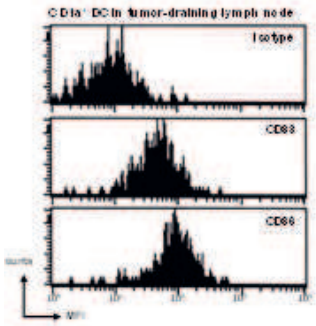
A. CD1a-positive cells derived from TDLN of patients with LABC treated with either GM-CSF or G-CSF as percentage of DC
B. Median Fluorescence Index (MFI) of CD83 and CD86 on CD1a-positive cells derived from TDLN of patients with LABC
C. Comparison of MFI on CD83 on CD1a-positive cells derived from TDLN of patients with LABC treated with either GM-CSF or G-CSF. * $P < 0.05$
D. Comparison of MFI on CD83 on CD1a-positive cells derived from TDLN of patients with LABC treated with either GM-CSF or G-CSF. * $P < 0.05$

A

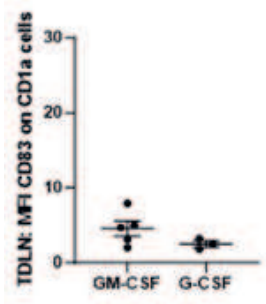


GM (mean ± SEM)	G (mean ± SEM)	p
1.38%±0.20	0.53%±0.21	0.018
N=7	N=5	

B

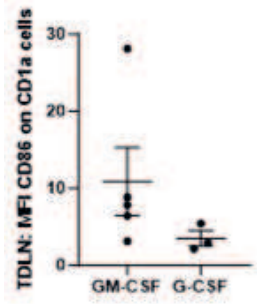


C



GM (mean ± SEM)	G (mean ± SEM)	p
4.54%± 1.00	2.50%±0.40	0.186
N=5	N=3	

D



GM (mean ± SEM)	G (mean ± SEM)	p
10.84%± 4.42	3.47%±1.7	0.261
N=5	N=3	

Discussion

Here we present the long-term survival data of a subset of patients with LABC included in the Spinoza study. Unfortunately, the study was prematurely terminated during its enrolment phase due to unforeseen shortage of GM-CSF. We present survival data of 34 patient accrued in two Dutch medical centres, immune monitoring was done in 16 patients of these patients.

BC specific mortality is dependent on stage, expression of receptors, histologic grade, nodal status and received systemic treatment (33). BC specific mortality has evolved over the years, at the time of inclusion in the Spinoza trial, 5- and 10-year OS in the Netherlands for BC stage II was 89% and 76% respectively, while for BC stage III 5- and 10-year OS was 67% and 46% (34). In the present study, including large 7 cm tumors and predominantly stage III disease, relatively high survival rates were observed (5- and 10-year OS of 71% and 56%) with a taxane free regimen, while neoadjuvant taxane containing regimens would become standard in the following years. The addition of taxanes yields a 17% reduction of hazard ratio for both DFS and OS (35). Especially the GM-CSF group seemed to have the best survival (trend), despite the fact that there was a higher percentage of pCR in the G-CSF group and the observed similar systemic immune modulating effects in both growth factors treatment groups. GM-CSF administration led to more profound loco-regional effects, exemplified by higher frequencies of mature $CD1a^+$ cDCs in TDLN in GM-CSF treated patients. One of the major mechanisms employed by tumors to escape from immune surveillance is hampered DC differentiation (36). Frequencies of circulating cDC have been reported to be significantly lower in a broad variety of cancer types, including patients with BC, as compared to healthy individuals (26, 37). Consistently, accumulation of immature and functionally impaired cDC has been documented in blood, tumors, and TDLN of patients with cancer and found to be a poor prognostic factor (38, 39). In evaluating the efficacy of NAC, assessing the immunological response in TDLN, and especially DCs, is a recognized prognostic factor (40, 41). The stronger $CD1a^+$ migratory DC-mobilizing effect of GM-CSF has also been observed in sentinel lymph nodes of patients with melanoma, BC and lymphoma (42-44). As GM-CSF augments the recruitment and activation of DCs, this could also impact the local immune response and induce T cell activation in the tumor microenvironment (TME) (45, 46).

Unfortunately our study did not include analysis of these tumor infiltrating lymphocytes (TILs). In contrast to the potentiating effects of GM-CSF on DC in TDLN, G-CSF was previously reported not to affect peripheral DC mobilization in patients with BC (n=10) and non-Hogkin's lymphoma (n=7) (47), which is in line with our current observations in BC.
In the present study, NAC combined with GM-CSF led to non-significant systemic increments of cDC1 and cDC2, while NAC combined with G-CSF led to non-significant systemic increments of cDC1 and significant increments in cDC2 and also pDC. Albeit the effect in the GM-CSF arm is not statistically significantly different than G-CSF, the magnitude of effect on pDC is an interesting observation. While pDC can contribute directly to the priming of anti-tumor effector CTL, either through type-I IFN production or through antigen presentation, cDC are generally found to be more powerful in this regard. cDC1 are suggested to be involved and superior in cross-tolerance or cross-priming of type-1 IFN responses, though mostly based on murine studies (48). cDC2 have been shown to secrete high levels of the type-1 T cell-skewing cytokine IL-12 upon appropriate stimulation (49). Previously, G-CSF treatment was shown to increase pDC and left cDC unchanged in healthy volunteers (50), in our study in patients with LABC, G-CSF based treatment correlated to an increase of both pDC and cDC, possible due to dose- or cancer-related response. The targeted effect of GM-CSF on cDC1 and cDC2, while pDC were left unchanged, may thus favour the generation of effective anti-tumor immunity and may have contributed to the improved OS observed in the GM-CSF treated population.
Disturbed cDC differentiation in patients with cancer is accompanied by an expansion of immature myeloid cells that under the influence of tumor-derived factors can convert into MDSC. These MDSC accumulate in tumors and are powerful suppressors of cell-mediated anti-tumor immunity. The disturbed balance between DC maturation and MDSC development can also contribute to the induction of highly suppressive Tregs (51). GM-CSF can act as a double-edged sword as it can both promote immunostimulatory effects, as well as exert tumor suppressive effects. GM-CSF recruits DC from the bone marrow (20) and DC differentiation is said to be promoted by GM-CSF-induced JAK2-STAT5 and MEK/ERK signaling (52). GM-CSF induces enhanced production of multiple cytokines, including IL-1, TNF and IL-6 leading to a further immunostimulatory proliferation of B and T lymphocytes (52, 53).

Excessive, sustained doses of GM-CSF have been shown to cause an immunosuppressive environment generating MDSC and causing activation of Tregs (49, 52-54). While we did observe the latter in both treatment arms, we only observed upregulation of systemic mMDSC rates on treatment with G-CSF. Although, differences were small and not statistically significant, this upregulation of mMDSC by G-CSF may also have contributed to the reduced OS in the G-CSF treated population. GM-/ G-CSF have been shown previously to increase levels of mMDSC in both patients with BC and melanoma (55, 56). Tregs have been described to be elevated in cancer patients and have been applied as an independent prognostic factor to identify patients with a high risk of relapse (57). In the current study, Tregs were measured based on CD4 and CD25 expression, as FoxP3 antibodies were not readily available at the time these measurements were done. Nevertheless, we have since confirmed by additional analyses incorporating FoxP3, CTLA4, CD45RA, and CD127 that the gating method employed in this study specifically defines activated Tregs (28). Although we found baseline Treg rates to be associated with OS, further upregulation did not correlate with OS or DFS, calling into question the suppressive functionality of these induced Tregs.

Of note, this study was performed in an era in which NAC or the use of taxanes in the curative setting were not standard, and dose levels of chemotherapy administered in this study were higher than currently applied. Moreover, testing for Her2-status was not yet standard-of-care. It is known that, compared to other LABC subtypes, significantly higher numbers of patients with a Her2+ or TN subtype achieve pCR after NAC (58). Moreover, it has been postulated that Her2+, HR+ and TN LABC have a different immune profile (59). Assessment of differences in survival and immune response between LABC subtypes after GM-CSF or G-CSF administration would have been interesting, but could not reliably be performed because of small patient numbers. Other factors which would had been interesting to study in larger patient numbers are the differences between tumor stage, dose-reductions and missed treatments as these differences might have influenced outcomes.

Since TILs have been demonstrated to be present and responsive to immunotherapeutic approaches in BC, there has been a surge in neoadjuvant immunotherapy trials in recent years (17). Our study illustrates changes that can be induced in myeloid and T cell subsets in peripheral blood and TDLN in response to exposure to GM-/G-CSF during NAC. The differential effect of GM-CSF on the immune system seems to be dose-dependent; systemic concentration and duration of exposure are key in predicting GM-CSF induced immunologic outcome (53, 56). Future strategies in LABC could include evaluation of local administration or activation of DCs; moreover, combining GM-CSF with CpG (60) could constitute an attractive regimen to aid the immune response. In melanoma, a comparable approach aimed at locally arming TDLN by administration of CpG-B has already shown promising results (28, 61). In the current era, GM-CSF sargramostim, could provide an alternative for administration of GM-CSF molgramostim and has shown some promising results in a phase 1-2 cancer vaccination strategy study for BC (62).

In conclusion, our study showed that GM-CSF recruited DCs may be able to process the tumor-derived antigens released from tumors upon exposure to chemotherapy, in this case doxorubicin and cyclophosphamide, and induce an effective and more robust anti-tumor immune response than current G-CSF based NAC strategies. Characteristics of the host immune system and tumor immune phenotype may also play a role on clinical outcomes (63). Analyzing chemotherapy effects on immune cell subsets in blood and in TME could help selecting the best chemotherapy partners in the immunotherapy development for patients with LABC.

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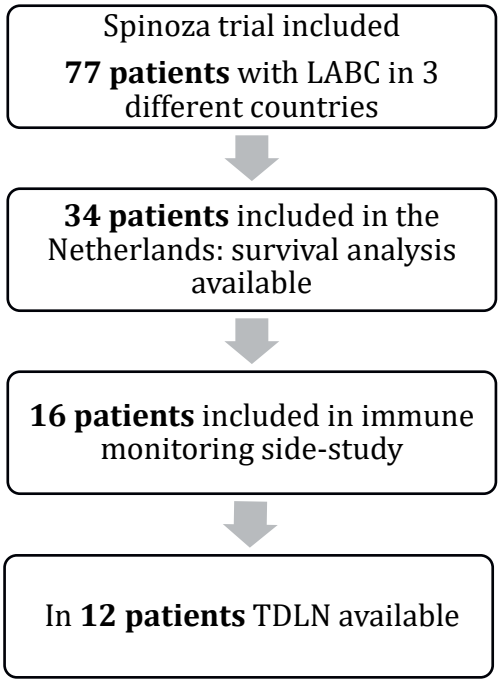
Supplemental Table 1: Baseline characteristics of 16 patients in immune monitoring side study

Patient Characteristics	All (n)	GM-CSF (n)	G-CSF (n)	p-value GM- vs G-CSF
Number of patients (n)	16	9	7	
Mean age (years)	49	53.2	49.7	0.604[a]
Histology				
-Ductal	50% (8)	44% (4)	57% (4)	>0.999[b]
-Lobular	44% (7)	44% (4)	43% (3)	>0.999[b]
-Other	6% (1)	11% (1)	0% (0)	>0.999[b]
Receptor status				
-HR+/Her2 unknown	31% (5)	22% (2)	43% (3)	0.596[b]
-HR+/Her2-	25% (4)	22% (2)	29% (2)	>0.999[b]
-HR+/Her2+	12.5% (2)	11% (1)	14% (1)	>0.999[b]
-HR-/Her2 unknown.	19% (3)	33% (3)	0% (0)	0.213[b]
-TN	6% (1)	0% (0)	14% (1)	0.438[b]
-Unknown	6% (1)	11% (1)	0% (0)	>0.999[b]
Stage				
-IIb	19% (3)	22% (2)	14% (1)	>0.999[b]
-IIIa	62.5% (10)	67% (6)	57% (4)	>0.999[b]
-IIIb	18.5% (3)	11% (1)	29% (2)	0.550[b]
Mean size BC (mm)	73	66	82	0.771[a]
Mean number of tumor-positive lymph nodes	4	3	5	0.534[a]
Tumor response to neo-adjuvant therapy (n)	6% (1)	11% (1)	0% (0)	>0.999[b]
-cCR	75% (12)	67% (6)	86% (6)	0.585[b]
-cPR	12.5% (2)	11% (1)	14% (1)	>0.999[b]
-pCR				
mOS (years)	Not reached	Not reached	Not reached	0.675[c]
5 year DFS	75% (12)	80% (8)	40% (4)	0.604[b]
10 year DFS	68.8% (11)	70% (7)	40% (4)	>0.999[b]
5 year OS	75% (12)	80% (8)	40% (4)	0.604[b]
10 year OS	68.8% (11)	70% (7)	40% (4)	>0.999[b]

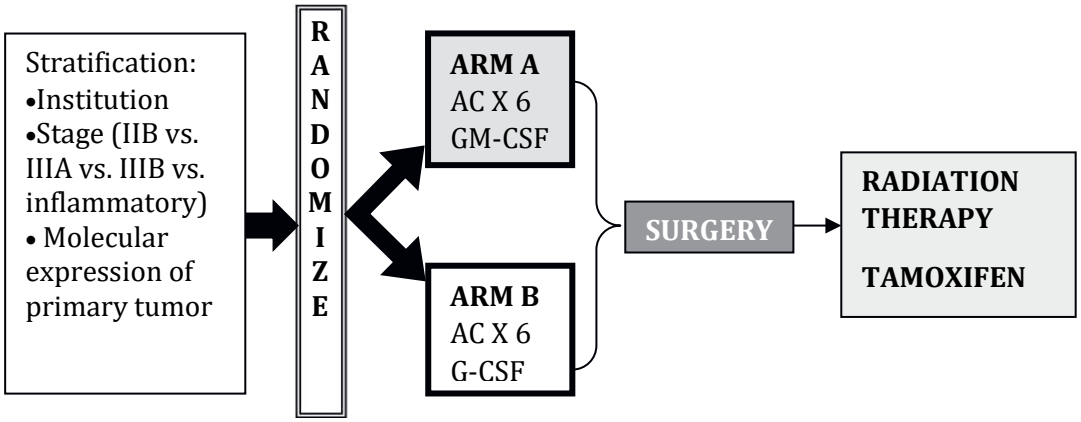
[a]unpaired T-test [b]Fisher's exact test [c]Log-Rank Mantel-Cox

Supplementary Figure 1: Inclusion of patients



Supplementary Figure 2: Study design and doses of chemotherapy in each cycle

Study Design A randomized, multicenter phase III study. Patients were randomized for treatment arm A (GM-CSF) or B (G-CSF).



Doses and regimen: The combination of doxorubicin and cyclophosphamide administered every 3 weeks for a total of 6 cycles to all patients and consisted of the following chemotherapy on day 1:

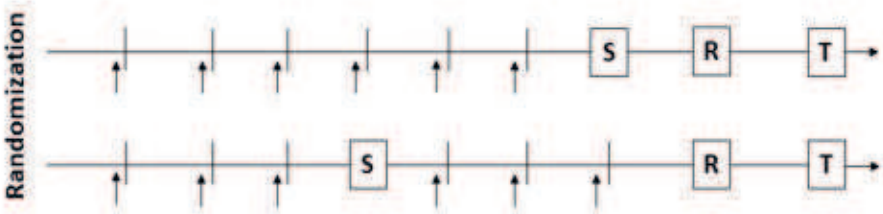
cycle	Doxorubicin (Adriamycin®) (mg/m² iv)		Cyclophosphamide (mg/m² iv)	
1	**90**	90	**1000**	1000
2	**82.5**	60	**875**	600
3	**82.5**	60	**875**	600
4	**75**	60	**750**	600
5	**75**	60	**750**	600
6	**75**	60	**750**	600
	Spinoza dose		**Spinoza dose**	

and

GM-CSF (Leukine®): 250 μg/m² daily sc (D2-12) (A) or

G-CSF (Neupogen®): 5 μg/kg daily sc (D2-12) (B)

Supplementary Figure 3



↑ Blood withdrawal for DC monitoring side study

Administration of chemotherapy (day 1) and G-CSF or GM-CSF (days 2-12)

S Modified radical mastectomy and axillary lymph node dissection

R Radiotherapy of the chest wall, axilla, supraclavicular region

T Hormonal treatment: tamoxifen 20mg/day, for 5 years, if HR+

Chapter 5 Systemic Therapy for Patients with HER2-Positive Breast Cancer and Brain Metastases: A Systematic Review and Meta-Analysis

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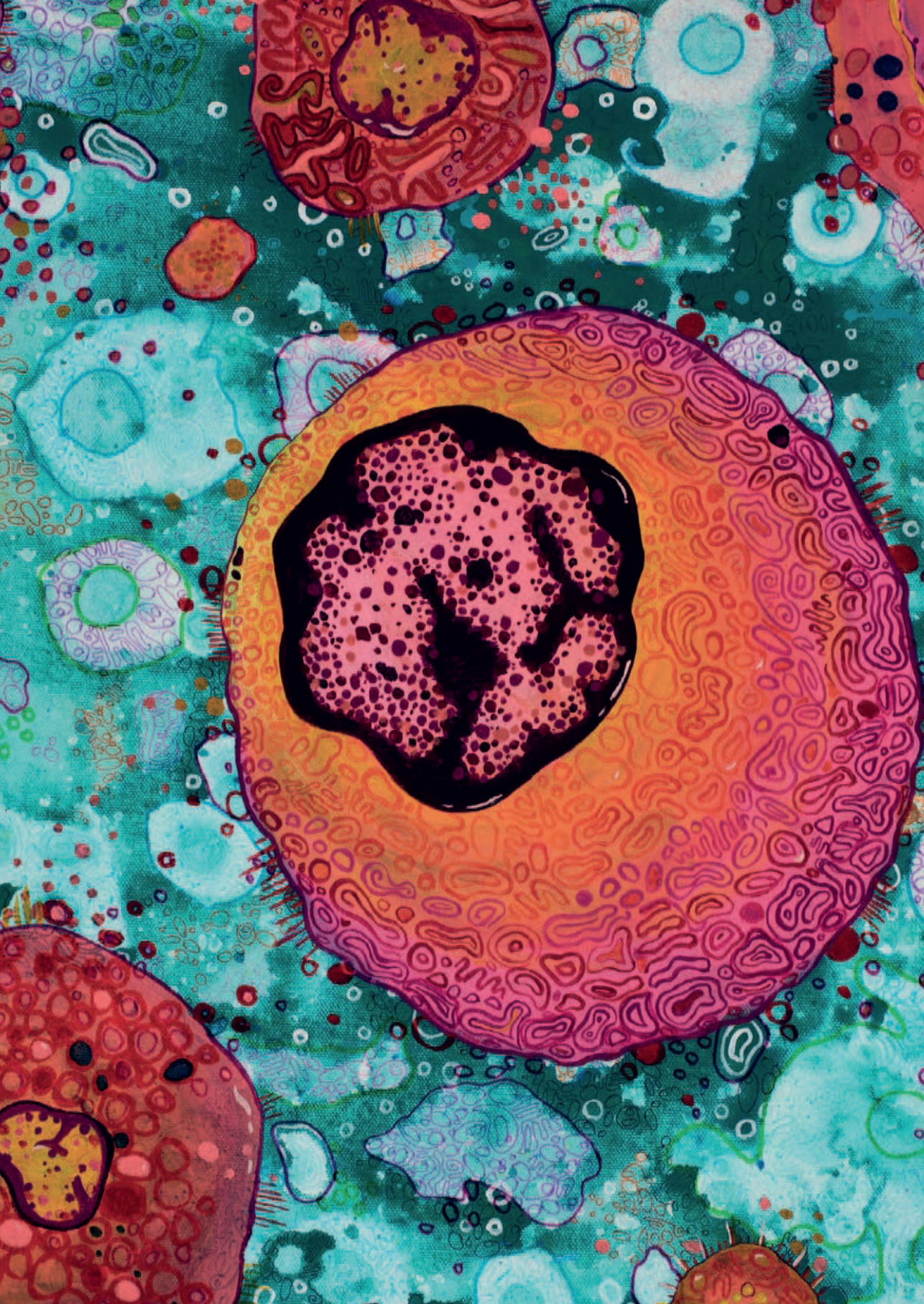
Cancers (Basel). 2022 Nov 15;14(22):5612.



Abstract

Aim: Patients with HER2-positive (HER2+) metastatic breast cancer (mBC) develop brain metastases (BM) in up to 30% of cases. Treatment of patients with BM can consist of local treatment (surgery and/or radiotherapy) and/or systemic treatment. We undertook a systematic review and meta-analysis to determine the effect of different systemic therapies in patients with HER2+ mBC and BM.

Methods: A systematic search was performed in the databases PubMed, Embase.com, Clarivate Analytics/Web of Science Core Collection and the Wiley/Cochrane Library. Eligible articles included prospective or retrospective studies reporting on the effect of systemic therapy on objective response rate (ORR) and/or median progression free survival (mPFS) in patients with HER2+ mBC and BM. The timeframe within the databases was from inception to 19 January 2022. Fixed-effects meta-analyses were used. Quality appraisal was performed using the ROBINS-I tool.

Results: Fifty-one studies were included, involving 3118 patients. Most studies, which contained the largest patient numbers, but also often carried a moderate-serious risk of bias, investigated lapatinib and capecitabine (LC), trastuzumab-emtansine (T-DM1) or pyrotinib. The best quality data and/or highest ORR were described with tucatinib (combined with trastuzumab and capecitabine, TTC) and trastuzumab-deruxtecan (T-DXd). TTC demonstrated an ORR of 47.3% in patients with asymptomatic and/or active BM. T-DXd achieved a pooled ORR of 64% (95% CI 43–85%, I^2 0%) in a heavily pretreated population with asymptomatic BM (3 studies, n = 96).

Conclusions: Though our meta-analysis should be interpreted with caution due to the heterogeneity of included studies and a related serious risk of bias, this review provides a comprehensive overview of all currently available systemic treatment options. T-Dxd and TTC that appear to constitute the most effective systemic therapy in patients with HER2+ mBC and BM, while pyrotinib might be an option in Asian patients.

Introduction

Metastatic breast cancer (mBC) is highly prevalent, 20% of mBC patients have HER2-positive (HER2+) mBC [1], 30% of which develop brain metastases (BM) [2]. This results in an incidence of BM in HER2+ mBC per patient-year of 13% [2]. Over the years, the survival of patients with HER2+ mBC and baseline BM improved significantly, from a median survival of 3–6 months to almost 30–38 months [3,4,5,6]. Patients who received anti-HER2 treatment had longer median OS than those without [7]. However, patients with BM still have a worse median survival compared to patients without BM [8]. Due to the blood-brain barrier (BBB) and the blood-tumor barrier (BTB), development of systemic treatments that are effective in patients with BM has been challenging, as large molecule biologic drugs supposedly have a limited ability to cross the (intact) BBB. The BBB is the term used to describe the unique characteristics of the endothelial cells of blood vessels that vascularize the central nervous system (CNS), which tightly regulates the movement of ions, molecules, and cells between the blood vasculature and the parenchyma, which is critical for neuronal function and protection [9]. The BTB describes the modifications to the BBB in patients with BM and primary brain tumors [9].

The cornerstone of the treatment of BM consists of local treatment modalities like surgery and/or stereotactic radiotherapy, often combined with systemic treatment. Besides a direct cytotoxic effect, systemic treatments can also exert a radio-sensitizing effect [10,11,12,13]. Systemic therapies for patients with HER2+ mBC include chemotherapy (e.g., taxanes), monoclonal antibodies (mAbs; eg. trastuzumab and pertuzumab (TP)), antibody-drug conjugates (ADCs; e.g., trastuzumab-emtansine (T-DM1), trastuzumab-deruxtecan (T-DXd)) and small molecule tyrosine kinase inhibitors (TKIs; e.g., Lapatinib, Pyrotinib, Neratinib, Afatinib, Cabozantinib and Tucatinib). Given the number of available therapies for patients with HER2+ mBC and the high prevalence of BM in these patients, it is important to understand which treatment is the most effective in terms of response rate and/or survival.

In addition to intracranial objective response rates (ORR), intracranial efficacy of a systemic treatment can also be deducted from its capacity to successfully postpone or prevent the development of BM.
The combination of TP and a taxane was investigated in the Cleopatra trial and demonstrated to be an effective first line therapy prolonging survival in HER2+ mBC [14,15]. Trastuzumab is a humanized mAb specific for extracellular domain IV of HER2. Pertuzumab is a humanized mAb specific for extracellular domain II of HER2, and thereby blocks a binding pocket necessary for receptor dimerization with HER3 [16]. While trastuzumab was considered not to cross the BBB due to its high molecular weight, it does appear to have intracranial efficacy, as it has been implicated to slow down the development of BM, and the use of trastuzumab is associated with a longer survival in mBC patients with BM [5,17]. Indeed, a study using ^{89}Zr-trastuzumab confirms that trastuzumab can access BM, possibly due to a compromised BBB [18]. Other imaging studies using ^{89}Zr-pertuzumab demonstrated that pertuzumab can also access BM, and similarly, ^{11}C lapatinib has also been shown to cross the BBB [19,20].
Since most patients with HER2+ mBC do not initially present with BM, they will probably have been treated with trastuzumab-based regimens before BM manifested. Currently used HER2 directed therapy in case of BM are mostly based on expert opinion, as patients with BM, especially symptomatic BM, were frequently excluded from trials. Though there have been earlier reviews on this subject [21,22,23,24], including one meta-analysis that focused on the combination of lapatinib and capecitabine (LC) in patients with BM of HER2+ mBC [25], our study, to the best of our knowledge, is the most complete overview comprising all different systemic therapies available to patients with HER2+ mBC and (a)symptomatic BM. Despite the high risk of bias and heterogeneity in the current meta-analysis, the data presented will support clinical decision making for these patients.

Methods

Search Strategy and Selection Criteria

This systematic review and meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A systematic search was performed in the databases PubMed, Embase.com, Clarivate Analytics/Web of Science Core Collection and the Wiley/Cochrane Library. The timeframe within the databases was from inception to 19th January 2022 and conducted by GB and IW. Eligible articles included prospective or retrospective studies reporting on the effect of systemic therapies on ORR and/or median progression free (mPFS) in patients with HER2+ mBC and BM. Studies were grouped based on investigational treatment arm, irrespective of active or inactive BM, treatment line, study design or quality. The search included keywords and free text terms for synonyms of 'breast neoplasm' combined with synonyms of 'HER2' combined with synonyms of 'brain metastases'. Reviews, animal studies, comments, letters, editorials, qualitative studies, case reports and case series (of less than 10 patients) were excluded from the search. A full overview of the search terms per database can be found in the supplementary information (see Tables S1–S4). No limitations on date or language were applied in the search. Selection of studies was done by two reviewers independently (IW and HV) based on title and/or abstract. Disagreement between reviewers was resolved by a third reviewer (WM).

Data Analysis

Data was extracted from published reports. Besides ORR and mPFS, data about intervention, line of therapy, previous local treatment, extra CNS disease, amount of BM and mOS was extracted if available, no assumptions were made in case of missing data. Meta-analysis was performed when a minimum of three studies reported similar effect measures for similar outcomes and similar interventions. Specifically, for the meta-analyses on mPFS and median overall survival (mOS), we needed months of survival and the respective confidence intervals. For the meta-analyses on ORR, we needed numbers of response and total numbers of the groups.

Summary estimates were computed by either using random-effects meta-analysis for the months of survival, or fixed-effects meta-analysis with Clopper-Pearson derived confidence intervals and Freeman-Tukey double arcsine transformation to stabilize inter-study variance for the ORR. Heterogeneity between studies was assessed by using the I^2 statistic, where we considered an I^2 value greater than 50% indicative of substantial heterogeneity. Subgroup analyses were not performed, due to low volume of studies. We performed sensitivity analyses if abstract-only articles were available, due to low quality of most included studies, we were not able to perform sensitivity analyses based on quality. When a meta-analysis was not possible because of a low number of studies, we used a descriptive synthesis. All analyses and plots were performed in RStudio version 4.0.3. using the 'meta' package [26].

We used the ROBINS-I tool to assess the quality of the included studies (non-randomized studies and RCTs) [27]. Additionally, we used domain 1 of the Risk of Bias 2 (RoB 2) tool (risk of bias arising from the randomization process) for the included RCTs [28]. This assessment was done at study level and performed by two independent reviewers (IW and WM). Disagreement between reviewers was resolved by a third reviewer (HV). Risk-of-bias plots were created using the robvis-tool [29].

Results

A flow diagram for the search strategy is shown in Figure 1. The search yielded 2686 studies, after deduplication, 1533 studies were identified, of which 1368 were excluded based on title and/or abstract. Reasons for exclusion were type of study (reviews, preclinical studies, phase 1 studies and studies comprising <10 patients) or the subject of the study (no HER2+ mBC, no patients with baseline BM, outcome not specifically related to type of systemic treatment and studies on biomarkers and genes and studies investigating local treatments). The 165 studies were discussed more thoroughly by the two reviewers, leading to 51 relevant articles involving 3118 patients included in the systematic review. Characteristics of the included studies are shown in Table 1 (BEEP, afatinib, neratinib, everolimus, cabozantinib, tucatinib, T-Dxd and trastuzumab/pertuzumab), Table 2 (T-DM1), Table 3 (lapatinib) and Table 4 (pyrotinib).

Figure 1. Flow chart of the search strategy.

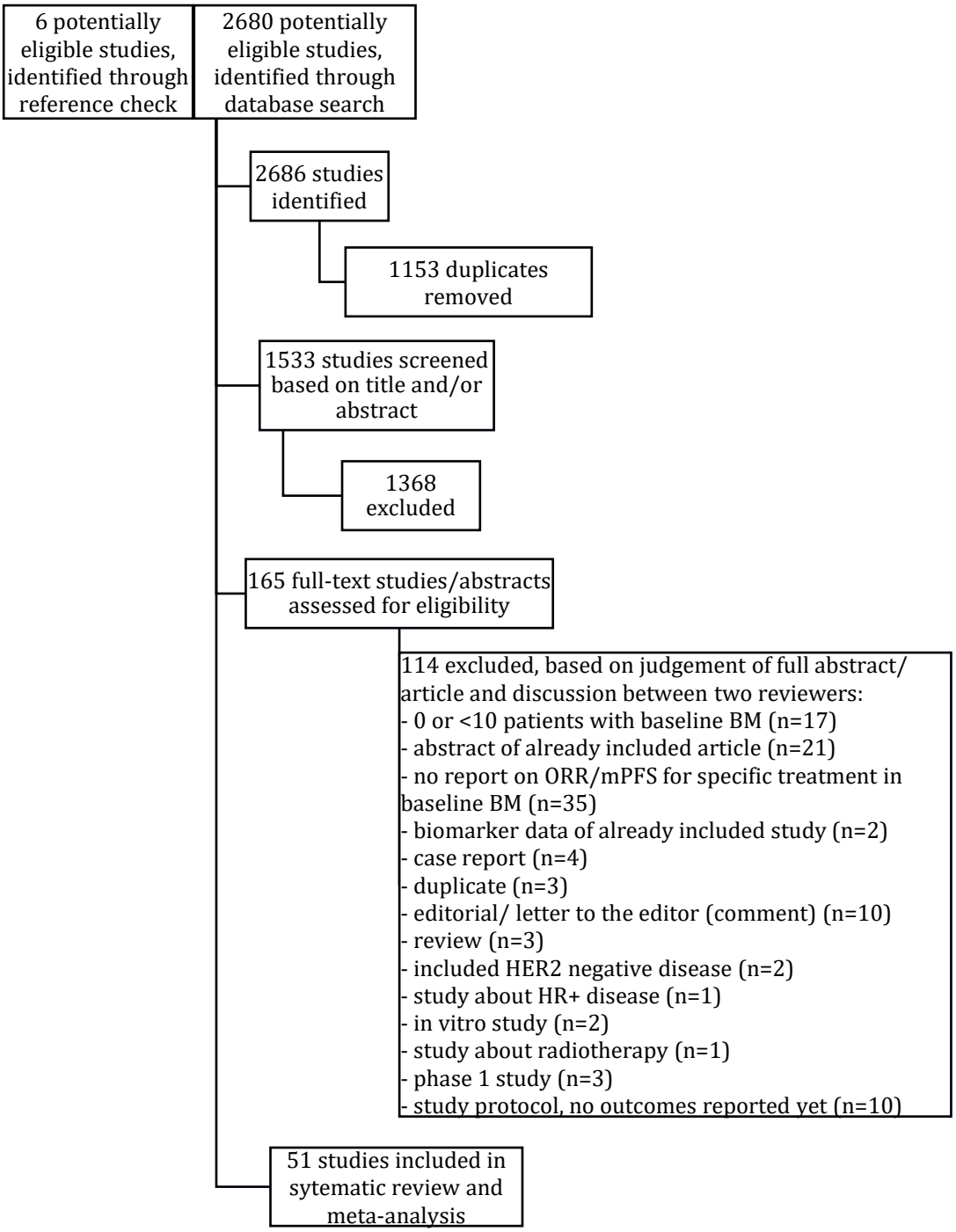


Table 1: Characteristics of various included studies (N=15)

Study	Phase	Patients with baseline BM (n)	Intervention (n)	Control (n)	Line of therapy	Previous local treatment for BM (%)	Extra CNS disease (%)	Amount of BM	mPFS (months)	mOS (months)	CNS ORR %
Lu 2015	phase 2	23	BEEP (Bevacizumab, Etoposide, Cisplatin)	Single arm	median 3 (Range 1-8)	100%	94.3%		7.7 (95% CI 6.6-8.8)	11.8 (95% CI 7.0-16.6)	69.6%
Cortes 2015-Lux Breast 3	phase 2, randomised	38	Afatinib + Vinorelbine (38)	Investigator choice (43) or Afatinib (40)	1-2 31%; 3-4 68%	83%	41%	59% > 3	2.8	8.6	8.0%
Freedman 2016	phase 2	40	Neratinib	Single arm	0-2 17%; 3-4 83%	100%			1.9	8.7	8.0%
Freedman 2019	phase 2	49	Neratinib + Capecitabine	Single arm	0 22%; 1 45%; ≥2 33%	92%	78%		5.5 (Range 0.8-18.8)	13.3 (Range 2.2-27.6)	49.0%
Hurvitz 2021-NALA	phase 3b (posthoc)	51	Neratinib + Capecitabine (51)	Lapatinib + Capecitabine (50)	2 68%; ≥3 32%	80%	84%		5.6 (95% CI 3.7-7.1)	13.9 (95% CI 8.9-17.5)	28.6%
Swearingen 2018	phase 2	32	Everolimus + Trastuzumab + Vinorelbine	Single arm	median 2 (Range 0-7)	97%	66%		3.9 (95% CI 2.3-5.0)	12.1 (95% CI 6.8-12.4)	4.0%
Hurvitz 2018	phase 2	19	Everolimus + Lapatinib + Capecitabine	Single arm	median 2.5 (Range 0-11)	63%	42%		6.2	24.2	28.0%
Leone 2020	phase 2	21	Cabozantinib + Trastuzumab	Single arm	median 3 (Range 1-7)	81%	>48%		4.1 (95% CI 2.8-6.2)	13.8 (95% CI 8.2-NR)	5.0%
N.Lin 2020-HER2Climb	phase 3	198	Tucatinib + Trastuzumab + Capecitabine (198)	Trastuzumab + Capecitabine (93)	median 3 (Range 1-14)	87%	97%		9.9 (95% CI 8.0-13.9)	18.1 (95% CI, 15.5-NR)	47.3%
Modi 2021-Destiny-Breast01	phase 2	24	Fam-Trastuzumab deruxtecan	Single arm	median > 6			median 5	18.1 (95% CI 6.7-18.1)	NR	58.0%
Bartsch 2021-Tuxedo 1	phase 2	10	Fam-Trastuzumab deruxtecan	Single arm	70% >2	60%					83.3%
Cortes 2022- Destiny Breast-03	phase 3	62	Fam-Trastuzumab deruxtecan (62)	Trastuzumab-emtansine (52)	2 50%; 3 22%; >5 8%				15.0 (95% CI 12.6-22.2)		63.0%
Lin 2021-PATRICIA	phase 2	39	High dose Trastuzumab/ Pertuzumab (+28% Other)	Single arm	median 3 (Range 2-5)						11.0%
Bergen 2021	retrospective	26	Trastuzumab/ Pertuzumab (60% + Chemo/ Local Therapy)	Single arm	median 1 (Range 1-6)		80%		8.0 (Range 1.0-55.0)	44.0 (range 2.0-61.0)	92.9%
Gamucci 2019- RePer	retrospective	21	Trastuzumab/ Pertuzumab+ taxane	Single arm	Median 1	48%			20 (95% CI 13-27)		52.4%

Table 2: Characteristics of included Trastuzumab-emtansine (T-DM1) studies (n=10)

Study	Phase	Patients with baseline BM (n)	Intervention (n)	Control (n)	Line of therapy	Previous local treatment for BM (%)	Extra CNS disease (%)	Amount of BM	mPFS (months)	mOS (months)	CNS ORR %
Krop 2015-Emilia	phase 3b (posthoc)	45	Trastuzumab-emtansine (45)	Lapatinib + Capecitabine (50)	median 3 (Range 1-13)	70%	79%		5.9	26.8	
Bartsch 2015	case series	10	Trastuzumab-emtansine	Single arm	1 40%; 2 60%	80%	90%	50% > 3	5.0 (95% CI 3.7-6.3)	8.5	30.0%
Yardley 2015	open label, prospective	26	Trastuzumab-emtansine	Single arm	median 8 (Range 3-23)				6.9 (95% CI 2.7-12.3)		27.3%
Mailliez 2016	retrospectief	14	Trastuzumab-emtansine	Single arm	median 2 (Range 0-7)				2.4 (Range 2.0-9.4)	9.1 (Range 3.7-24.8)	28.0%
Jacot 2016	retrospectief	39	Trastuzumab-emtansine	Single arm	median 2 (Range 0-8)	95%	82%	median 2 (Range 1-11)	6.1 (Range 5.2-18.3)	NR	44.0%
Okines 2018	retrospectief	16	Trastuzumab-emtansine	Single arm	median 2 (Range 0-6)	100%			9.9 (95% CI 3.9-12.2)	15.3 (95% CI 4.7-NR)	
Fabi 2018	retrospectief	87	Trastuzumab-emtansine	Single arm	1-2 51%; 3-4 49%	100%		25% > 3	7.0 (95% CI 5.4-8.6)	14.0 (95% CI 12.2-15.8)	25.0%
Montemurro 2019-Kamilla	phase 3b (posthoc)	398	Trastuzumab-emtansine	Single arm	0-2 48%; 3-4 31%; ≥5 19%	47%	79%		5.5 (95% CI 5.3-5.6)	18.9 (95% CI 17.1-21.3)	21.4%
Bahceci 2021	retrospectief	87	Trastuzumab-emtansine	Single arm					9.0	19	
Cortes 2022-Destiny Breast-03	phase 3b (posthoc)	52	Trastuzumab-emtansine (52)	Fam-Trastuzumab deruxtecan (62)	2				5.7 (95% CI 2.9-7.1)		34.0%

Table 3: Characteristics of included Lapatinib and/or Capecitabine studies (n=20)

Study	Phase	Patients with baseline BM (n)	Intervention (n)	Control (n)	Line of therapy	Previous local treatment for BM (%)	Extra CNS disease (%)	Amount of BM	mPFS (months)	mOS (months)	CNS ORR %
Lin 2008	phase 2	39	Lapatinib	Single arm	1-2 25%; ≥3 75%	95%	>62%		3.0 (95% CI 2.3-3.7)	7	2.6%
Lin 2009	phase 2	242	Lapatinib	Single arm	1-2 56%; 3-4 43%; ≥5 11%	95%			2.4 (95% CI 1.9-3.3)	6.4 (95% CI 5.5-8.3)	6.0%
Wang 2021	retrospective	42	Lapatinib	Single arm	1 17.4%; 2 53.9%; 3 20.1%; ≥4 7.8%	59%			6.3 (95% CI 5.1-7.5)		31.0%
Gavilá 2019	retrospective	38	Lapatinib + Trastuzumab	Single arm	3 (2-4)				3.8	15.2	
Boccardo 2008	open label, prospective	138	Lapatinib + Capecitabine	Single arm	≥2 100%						18.0%
Lin 2009*	phase 2 (expansion)	50	Lapatinib + Capecitabine	Single arm	2	95%			3.7 (95% CI 2.4-4.4)	NR	20.0%
Sutherland 2010	open label, prospective	34	Lapatinib + Capecitabine	Single arm	mean 2.4 (Range 1-5)	94%			5.1 (95% CI 3.5-6,5)	NR	21.0%
Metro 2011	retrospective	30	Lapatinib + Capecitabine	Single arm	median 2 (Range 1-5)	87%	97%	40% > 3	5.1 (95% CI 2.6-7.5)	11 (95% CI 4.3–17.6)	32.0%
Lin 2011	phase 2, randomised	13	Lapatinib + Capecitabine (13)	Lapatinib + Topotecan (9)	>1	100%	59%		NR	NR	38.5%
Cetin 2012	retrospective	85	Lapatinib + Capecitabine	Single arm	>3 74.1%	100%	96.5%		7.0 (95% CI 5.0-10.0)	13 (95% CI 9-17)	27.1%
Bachelot 2013-LANDSCAPE	phase 2	44	Lapatinib + Capecitabine	Single arm	1-2 78%; 3-4 22%	0%	84%	median 3 (Range 1-25)	5.5 (95% CI 4.3-6.0)	17 (95% CI 13.7-24.9)	57.0%
Ro 2012	open label, prospective	58	Lapatinib + Capecitabine	Single arm	>3 38%	91%			4.5 (95% CI 4.2-5.5)	12.2 (9.9-14.5)	15.0%
Dubianski 2014	retrospective	19	Lapatinib + Capecitabine	Single arm					8.1		
Shawky 2014	phase 2	21	Lapatinib + Capecitabine	Single arm	>2 100%	76%	91%	57% > 3	5.5 (Range 1.1–22.0)	11	33.3%
Krop 2015-Emilia	phase 3b (posthoc)	50	Lapatinib + Capecitabine (50)	Trastuzumab-emtansine (45)	median 3 (Range 1-13)	70%	79%		5.7	12.9	
Kaplan 2014	retrospective	46	Lapatinib + Capecitabine	Single arm	> 2 48.9%	96%	86.5%	48% > 3		19.1	36.8%
Gui 2020	retrospective	14	Lapatinib + Capecitabine	Single arm	> 3 82.6%	100%			8.4 (95% CI 2.2 - 14.7)		35.7%
Seligmann 2020-LANTERN	phase 2, randomised	16	Lapatinib + Capecitabine (16)	Trastuzumab + Capecitabine (14)		100%	70%		6.2 (95% CI 3.6-7.1)	NR	25.0%
Hurvitz 2021-NALA	phase 3b (posthoc)	50	Lapatinib + Capecitabine (50)	Neratinib + Capecitabine (51)	2 68%; ≥3 32%	80%	84%		4.3 (95% CI 2.8-5.6)	12.4 (95% CI 9.7–16.9)	28.2%
Yang 2021	retrospective	25	Lapatinib + Chemo (71% Capecitabine)	Pyrotinib + Chemo (80% Capecitabine)					3.5		

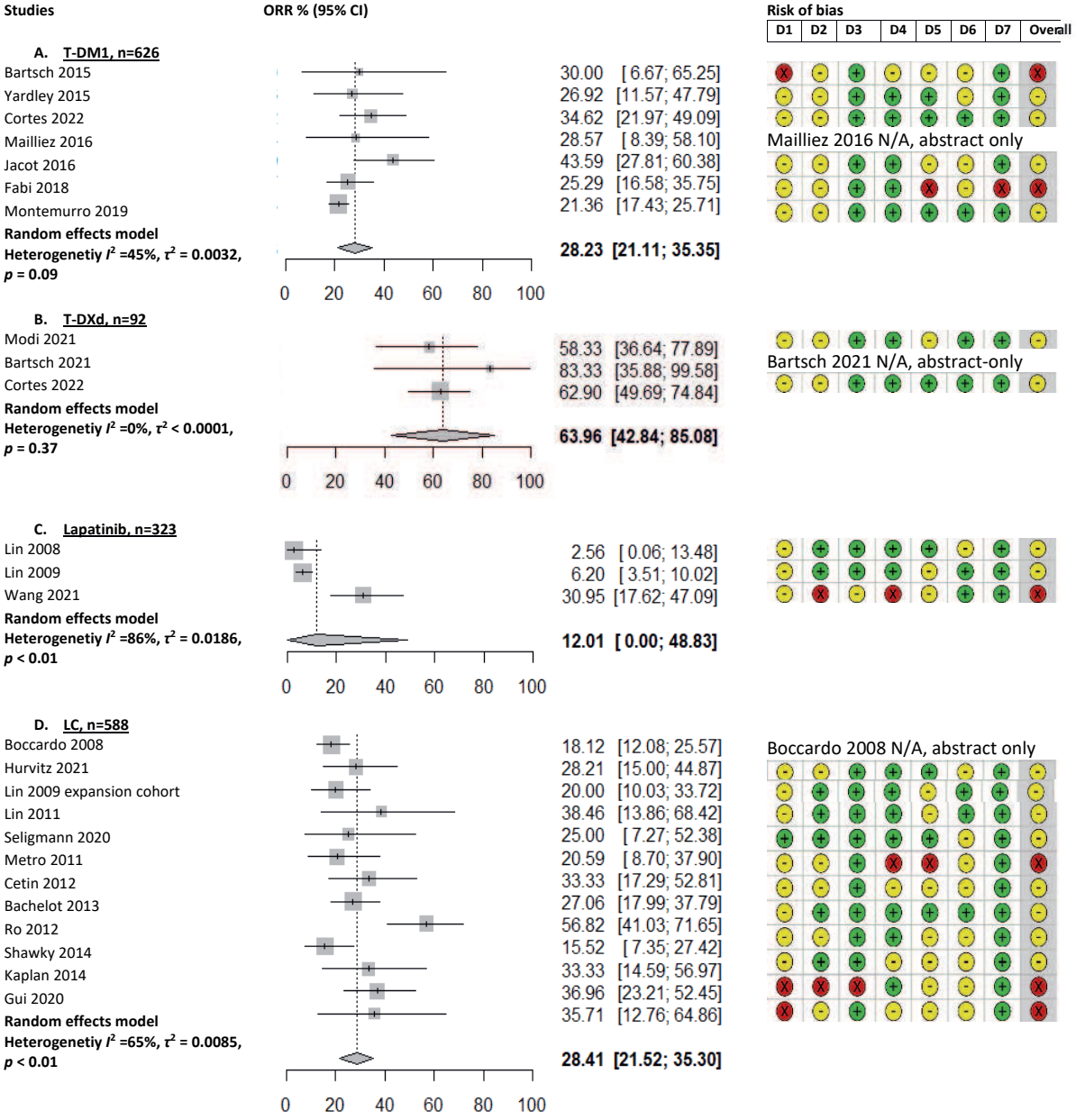
*expansion cohort of Lin 2009

Table 4: Characteristics of included Pyrotinib studies (n=9)

Study	Phase	Patients with baseline BM (n)	Intervention (n)	Control (n)	Line of therapy	Previous local treatment for BM (%)	Extra CNS disease (%)	Amount of BM	mPFS (months)	mOS (months)	CNS ORR %
Yan 2020- Phenix	phase 3	21	Pyrotinib + Capecitabine (21)	Capecitabine (10)					6.9 (95% CI 5.4-NR)		
Yan 2022- Permeate	phase 2	78	Pyrotinib + Capecitabine	Single arm		76%					66.7%
Y.Lin 2020	retrospective	31	Pyrotinib + Capecitabine (59%)/ Other(41%)	Single arm	1-2 38% 3 22% ≥4 40%	55%	88,50%		6.7 (Range 4.7-8.7)		28.0%
Gao 2021	retrospective	42	Pyrotinib (+ Chemo 59%)	Single arm	>1 93%	82%	90,00%	17% >5	11.1		47.6%
Zhang 2021	retrospective	21	Pyrotinib + Capecitabine (55%)/ Other (38%)/ Mono (7%)	Single arm	>1 88%				16.6 (95% CI 13.7 – 24.1)		45.5%
Yang 2021	retrospective	13	Pyrotinib + Chemo (80% Capecitabine) (13)	Lapatinib+ Chemo (71% Capecitabine) (35)					6.5		
Anwar 2021	retrospective	39	Pyrotinib + Capecitabine (64%)/ Other (36%)	Single arm	> 3 62%	43%			8.7 (95% CI 6.4-11.9)	13.9	28.0%
C.Li 2021	retrospective	53	Pyrotinib + Capecitabine (35%)/ Other (63%)/ Mono (3%)	Single arm		77%			7.0 (Range 6.1-7.8)		44.7%
Y.Li 2021	retrospective	23	Pyrotinib + Vinorelbine	Single arm					6.3 (Range 3.4-9.2)		

Of the 51 included studies, 4 studies were abstract-only studies. Consequently, there was not enough information for risk of bias interpretation. The other 47 articles comprised of 8 retrospective analysis of randomized studies; namely, 3 open label randomized phase 2 studies (Lux Breast3, Lantern and EGF107671), 3 open label randomized phase 3 studies (Emilia, NALA and Destiny Breast 03) and 2 double blind randomized phase 3 studies (Phoenix and HER2CLIMB). In addition, one open-label phase-3b single arm study was included (Kamilla). Further studies consisted of 14 single arm phase 2 studies, 1 case series, 4 open-label extended access program studies and 23 retrospective observational single arm studies. Risk of bias was assessed for all included studies (Figure 2 and Figure 3). A common cause of bias for many included studies resulted from the different criteria used for assessing progression of BM, and often this outcome was not a primary or secondary endpoint. T-DM1 and LC studies were mostly of moderate-serious risk of bias (Figure 2B,D). The pyrotinib studies were all of serious risk of bias, except for the Phoenix trial (Figure 2E). Especially the HER2CLIMB trial had a low risk of bias (Figure 3). Despite presenting a complete overview of all treatment options to date, the reader should realize that due to different trial designs (prospective, retrospective, randomized and non-randomized), different treatment lines and inclusion of both active and inactive BM, the presented meta-analysis was hampered by bias and heterogeneity.

Figure 2


Studies
ORR % (95% CI)
Risk of bias
D1 D2 D3 D4 D5 D6 D7 Overall
A. T-DM1, n=626
Bartsch 2015 30.00 [6.67; 65.25]
Yardley 2015 26.92 [11.57; 47.79]
Cortes 2022 34.62 [21.97; 49.09]
Mailliez 2016 28.57 [8.39; 58.10]
Jacot 2016 43.59 [27.81; 60.38]
Fabi 2018 25.29 [16.58; 35.75]
Montemurro 2019 21.36 [17.43; 25.71]
Random effects model 28.23 [21.11; 35.35]
Heterogenetiy I^2 =45%, τ^2 = 0.0032, p = 0.09
Mailliez 2016 N/A, abstract only
B. T-DXd, n=92
Modi 2021 58.33 [36.64; 77.89]
Bartsch 2021 83.33 [35.88; 99.58]
Cortes 2022 62.90 [49.69; 74.84]
Random effects model 63.96 [42.84; 85.08]
Heterogenetiy I^2 =0%, τ^2 < 0.0001, p = 0.37
Bartsch 2021 N/A, abstract-only
C. Lapatinib, n=323
Lin 2008 2.56 [0.06; 13.48]
Lin 2009 6.20 [3.51; 10.02]
Wang 2021 30.95 [17.62; 47.09]
Random effects model 12.01 [0.00; 48.83]
Heterogenetiy I^2 =86%, τ^2 = 0.0186, p < 0.01
D. LC, n=588
Boccardo 2008 18.12 [12.08; 25.57]
Hurvitz 2021 28.21 [15.00; 44.87]
Lin 2009 expansion cohort 20.00 [10.03; 33.72]
Lin 2011 38.46 [13.86; 68.42]
Seligmann 2020 25.00 [7.27; 52.38]
Metro 2011 20.59 [8.70; 37.90]
Cetin 2012 33.33 [17.29; 52.81]
Bachelot 2013 27.06 [17.99; 37.79]
Ro 2012 56.82 [41.03; 71.65]
Shawky 2014 15.52 [7.35; 27.42]
Kaplan 2014 33.33 [14.59; 56.97]
Gui 2020 36.96 [23.21; 52.45]
Random effects model 28.41 [21.52; 35.30]
Heterogenetiy I^2 =65%, τ^2 = 0.0085, p < 0.01
Boccardo 2008 N/A, abstract only
0 20 40 60 80 100

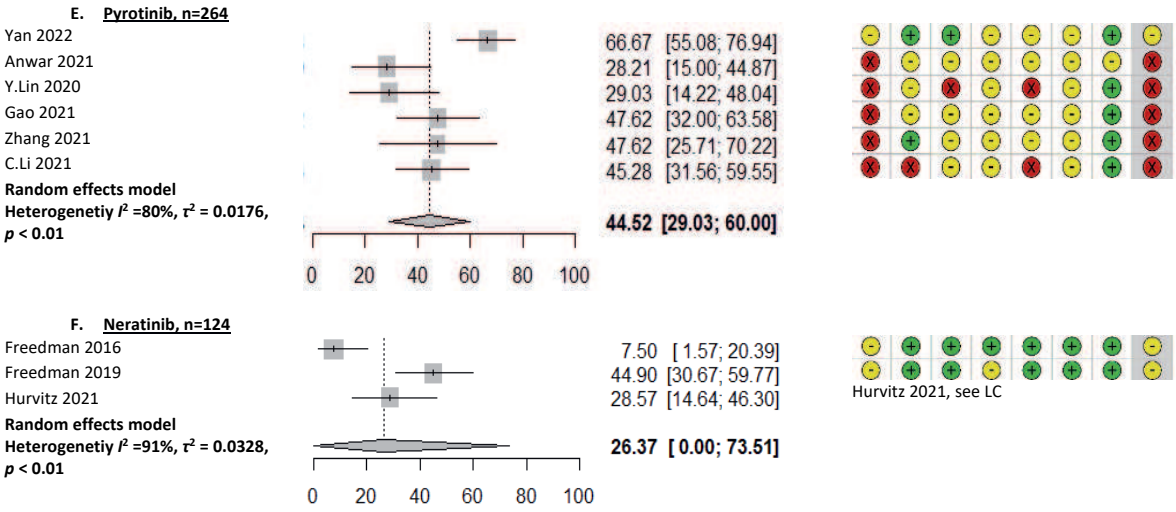
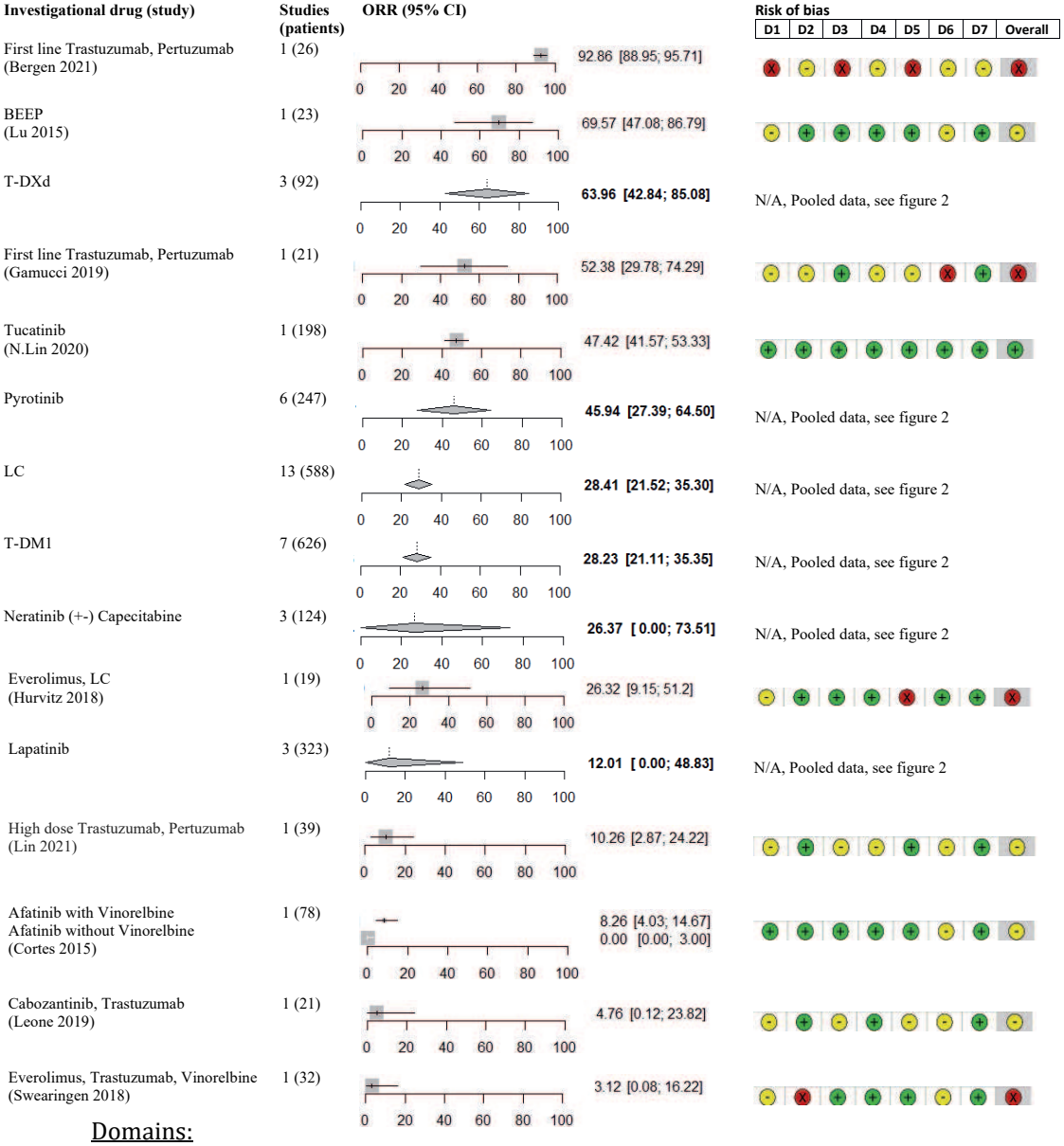


Figure 2. Pooled ORR meta-analysis per drug (combination) and quality assessment of risk of bias (**A**) Trastuzumab-emtansine (T-DM1); (**B**) Trastuzumab-deruxtecan (T-DXd); (**C**) Lapatinib; (**D**) Lapatinib + capecitabine (LC); (**E**) Pyrotinib; (**F**) Neratinib; * amount of patients receiving combination therapy with chemotherapy, mostly capecitabine (see **Table 4**).

Figure 3. ORR for all drug (combinations). Overview of single studies, pooled meta-analysis and quality assessment of risk of bias.



Domains:

D1: Bias due to confounding
D2: Bias due to selection of participants
D3: Bias in classification of interventions
D4: Bias due to deviations from intended interventions
D5: Bias due to missing data
D6: Bias in measurements of outcomes
D7: Bias in selection of reported results

Monoclonal Antibodies

Two studies, investigating 47 patients, assessed the efficacy of first line TP and a taxane (Table 1). In the first line setting, local treatment of BM is standard of care, so these results should be interpreted for the combination. In the subset of 21 patients with baseline (inactive) BM in the retrospective Reper study, an ORR of 52.4% was achieved (Figure 3) and a mPFS of 20 months (95% CI 13–27 months) [44]. The retrospective study by Bergen et al. [43] investigated the effect of different first-line systemic treatments for 252 patients with HER2+ mBC and BM. Of all included patients, 26 patients received first line TP combined with local therapy with or without chemotherapy, leading to an ORR of 92.9% (Figure 3), mPFS of 8.0 months (range 1–55 months) and mOS of 44 months (range 2–61 months). Both the Reper study as well as the study by Bergen et al. had a serious risk of bias (Figure 3) due to the retrospective design, no routine MRI scans of the brain and concomitant local therapies.

The single arm phase 2 PATRICIA study reported on high dose trastuzumab (HDT) (6 mg/kg weekly) in combination with pertuzumab after progression on standard dose trastuzumab and a median of three lines of previous therapy (n = 39) (Table 1) [42]. This was based on a preclinical mammary tumor graft model of HER2+ mBC, in which up to three times the regular dose of trastuzumab was needed to achieve similar responses in brain tumor grafts [79]. HDT was demonstrated to be safe but resulted in a low ORR of 11% (Figure 3).

Antibody Drug Conjugates

ADCs approved for the treatment of patients with HER2+ mBC are T-DM1 and T-DXd. T-DM1 contains the microtubule-inhibitory agent DM1 (derivative of maytansine) conjungated to trastuzumab [80]. T-DXd has the DNA topoisomerase I inhibitor deruxtecan conjugated to trastuzumab [81]. Compared to T-DM1, T-DXd has a higher antibody to drug ratio (8 versus (vs.) 3–4) and is probably more potent than T-DM1 as a result of the properties of its payload that facilitates penetration of deruxtecan through the cell membrane of the HER2+ tumor cells or neighboring cells, without requiring high HER2 expression levels [22,81].

T-DM1 was studied in 10 trials comprising 774 patients, mostly second line treatment (Table 2); 5 retrospective studies [48,49,50,51,53], 2 posthoc analyses of open label randomized phase 3 trials [41,45], 1 case series [46], 1 expanded access program [47] and 1 posthoc analysis of an open label single arm study [52] Pooled ORR was 28% (95% CI 21–35%; I^2 45%) and remained the same after excluding abstract-only articles in the sensitivity analysis (Figure 2A and Figure S1A). The Kamilla study demonstrated modest activity with an ORR of 21%. In this study, 6% of patients had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 2, and a relatively low number of patients received prior pertuzumab (4%) or local treatment for BM (47%) [52]. mPFS was similar in all studies with a pooled mPFS of 5.8 months (95% CI 5.1–6.6 months; I^2 42%) (Figure 4A). mOS was reported in seven studies with a median of 15.3 months (range 8.5–26.8 months) (Table 2).

T-DXd was studied in 3 trials and 96 patients (Table 1); 2 single arm phase 2 trials [39,40] and a sub-analysis of an open label randomized phase 3 trial [41]. These studies included heavily pretreated patients with BM (54% pretreatment with HER2 targeting TKIs, TP and taxanes). The pooled ORR of the three studies was 64% (95% CI 43–85; I^2 0%) (Figure 2B). Most patients had stable BM. Efficacy in patients with BM was not an endpoint of the phase 2 DESTINY-Breast01 and phase 3 DESTINY-Breast03 studies. The single arm phase two TUXEDO-1 trial included patients with active BM and is still actively recruiting patients; data of the first 10 patients showed a promising ORR of 83.3%. The phase 2 DESTINY-Breast01 and phase 3 DESTINY-Breast03 studies reported on mPFS, ranging 15.0–18.1 months for patients with asymptomatic BM. There were no reports on mOS.

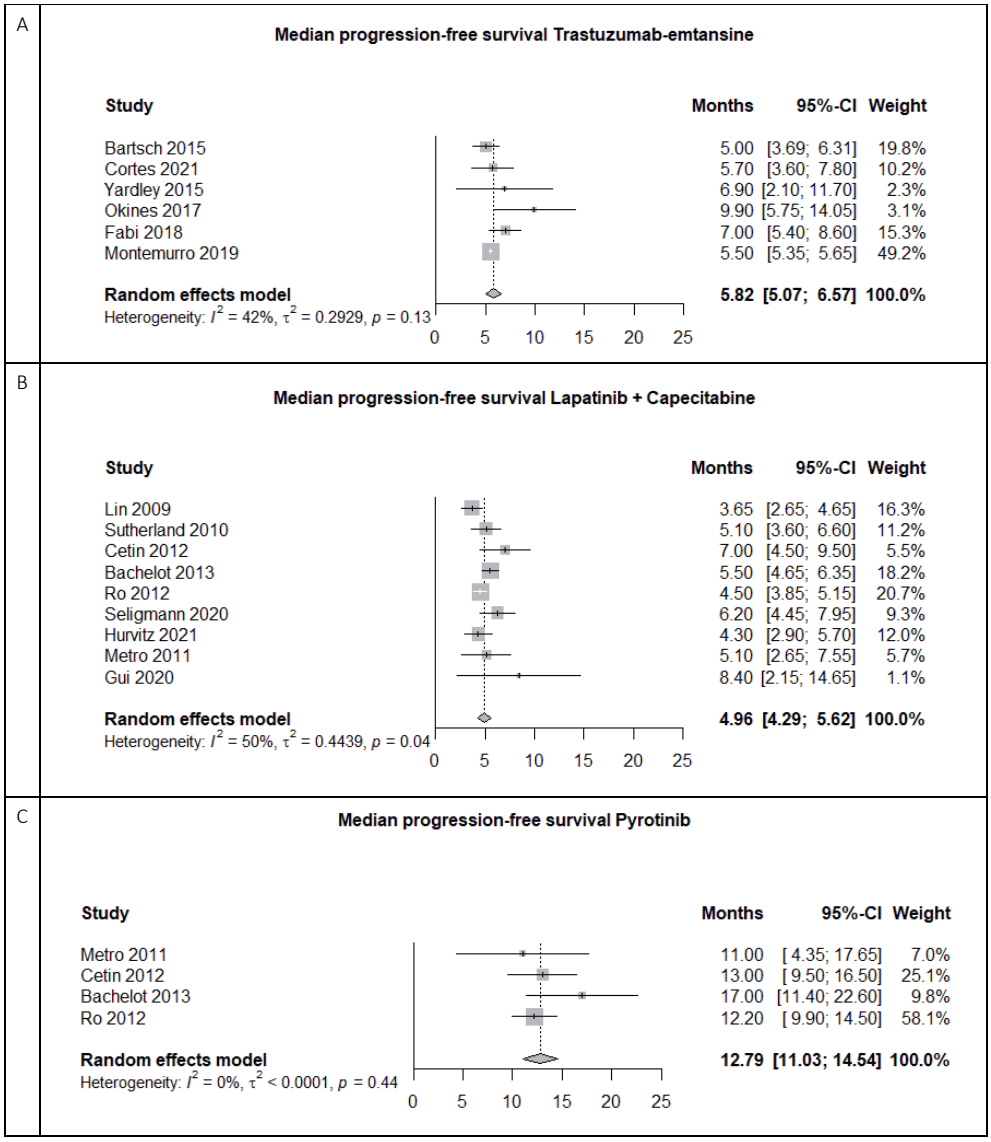


Figure 4. Pooled mPFS (months) meta-analysis; (**A**) Trastuzumab-emtansine (T-DM1); (**B**) Lapatinib + capecitabine (LC); (**C**) Pyrotinib.

Tyrosine Kinase Inhibitors

Several TKIs have been evaluated in patients with HER2+ mBC. These TKIs differ in molecular weight, selectivity and reversibility of binding to HER2-protein, efficacy and their safety profile. Lapatinib is a reversible dual inhibitor of HER1/EGFR and HER2 [82]. Pyrotinib, neratinib and afatinib are all irreversible inhibitors of HER1/EGFR, HER2 and HER4 [31,83,84]. Cabozantinib is a multi-TKI inhibiting MET, VEGFR2, RET and other TKIs [37]. Tucatinib is a reversible and highly selective HER2 inhibitor [85].

There are two phase 2 studies [54,55] and one retrospective study [56], comprising 323 patients addressing lapatinib monotherapy. These three studies led to a pooled ORR of 12% (95% CI 0–49%; I^2 86%) and mPFS of 3.0 months (range 2.4–6.3 months) (Figure 2C and Table 3). The retrospective study by Wang et al. included patients in Chinese centers and demonstrated a relatively high ORR of 31% and mPFS of 6.3 months, independent of the line of therapy [56]. Of note, 26% of patients in this study received lapatinib combined with trastuzumab, and 59% of patients had not been previously treated with HER2 directed therapies. Moreover, only 59% had been treated with local therapy for BM compared to 95% in both studies by Lin et al. 2008 and 2009 [54,55]. The combination of lapatinib and trastuzumab was studied in the retrospective Trastyvere study, patients with BM had 3.8 months of mPFS and 15.2 months of mOS [57].

A total number of 16 studies, including 693 patients combined, which investigated LC have been included in the meta-analysis; 2 randomized phase 2 studies [61,69], 2 single arm phase 2 studies [63,66], 1 expansion cohort of a single arm phase 2 study [55], 3 expanded access program studies [58,59,64], 6 retrospective studies [60,62,65,67,68,70] and 2 posthoc analyses of open label phase 3 trials (Table 3) [34,45]. This demonstrated a pooled ORR of 28% (95% CI 21–35%; I^2 62%). After excluding abstract-only articles, the pooled ORR remained the same (Figure 2D and Figure S1B). Of note, though the Landscape trial demonstrated a high ORR of 57% [63], a high percentage of 78% of patients in this study were treated with LC in first or second line and all included patients had previously untreated BM.

Survival analysis resulted in a pooled mPFS of 5.0 months (95% CI 4.3–5.6 months; I^2 50%) (Figure 4B) and a pooled mOS of 12.8 months (95% CI 11.0–14.5 months; I2 0%) (Figure S2).
In this meta-analysis, 9 studies investigating pyrotinib in a total of 321 Asian patients were included (Table 4); 1 double blind phase 3 study [71], 1 single arm phase 2 trial [78] and 7 retrospective studies [70,72,73,74,75,76,77]. Pooled ORR was 43% (95% CI 27–59%; I^2 80%) (Figure 2E). Most studies were of serious risk of bias due to retrospective design. Pyrotinib was mostly combined with capecitabine, but it was also given as monotherapy or in combination with other regimens. These studies were predominantly in second line, after trastuzumab-based therapy, patients had not received prior treatment with TP or T-DM1. Most studies did not report on previous local treatment for BM, and if reported, it was quite low in three studies (0%, 43%, 55%) (Table 4). Importantly, the phase 2 study by Yan et al. underscored the effect of prior radiotherapy for BM on ORR (radiotherapy naive cohort ORR of 74.6% vs. progressive disease after radiotherapy cohort ORR of 42.1%). Three studies were available for a pooled analysis of mPFS, which was 10.1 months (95% CI 4.3–15.8 months; I^2 88%) (Figure 4C). A mOS of 13.9 months was reported in one study; for the other studies, this information was lacking [75].
Neratinib was investigated as monotherapy in one phase 2 study (*n* = 40) [32] and in combination with capecitabine in two studies; a phase 2 study [33] and a posthoc analysis of a phase 3 trial [34] with a total of 100 patients (Table 1). Combining these three studies led to a heterogeneous meta-analysis due to difference in mono or combined intervention arms. In the neratinib monotherapy study, an ORR of 8% was demonstrated, while the two studies combining neratinib and capecitabine (NC) found an ORR of 29% and 49% (calculated from both lapatinib-naïve and lapatinib-treated cohort). Combining these three studies, a pooled ORR of 26% (95% CI 0–74%) was calculated (Figure 2F). For neratinib monotherapy, mPFS was 1.9 months vs. 5.5 and 5.6 months for NC. mOS was 8.7 months in the neratinib monotherapy study vs. 13.3 and 13.9 months for NC.

Afatinib was studied in one randomized phase 2 study as monotherapy (n = 40) and combined with vinorelbine (n = 38) [31]. Notably, in this study, only 41% of patients with BM also had extracranial disease (Table 1). Afatinib, alone or in combination, showed low efficacy with an ORR of 0% vs. 8% respectively (Figure 3) and a mPFS of 2.7 vs. 2.8 months, respectively. Due to low efficacy (and frequent adverse events), no further development of afatinib for HER2+ mBC is currently planned [31].

The combination of cabozantinib and trastuzumab was studied in one study with 21 heavily pretreated patients (Table 1) [37]. The investigators hypothesized that simultaneous targeting of both MET and VEGFR2 by cabozantinib might combine antivascular and anti-tumor activity. The ORR was 5% (Figure 3), mPFS 4.1 months (95% CI 2.8–6.2) and mOS 13.8 months (95% CI 8.2-NR). Cabozantinib therefore had insufficient activity and its use in this setting has not been further explored.

The combination of tucatinib, trastuzumab and capecitabine (TTC) was studied In 612 patients in the HER2CLIMB study [38]. A secondary endpoint of this double-blind randomized phase 3 trial was the efficacy of TTC in patients with (active and inactive) BM. Of the 612 patients, 291 patients had BM at baseline; 198 patients were treated with TTC, while 92 patients were treated with placebo, trastuzumab and capecitabine (Table 1, Figure 3). The ORR for TTC was 47.3% vs. 20.0% for placebo (p = 0.03). CNS mPFS for TTC was 9.9 vs. 4.2 months for placebo (HR 0.32; 95% CI 0.22–0.48; p < 0.0001) [85]. mOS for TTC was 18.1 vs. 12.0 months for placebo (HR 0.58; 95% CI 0.40–0.85; p = 0.005). Interestingly, 30 patients who had isolated CNS progression were allowed to continue systemic treatment according to the study protocol, after receiving local CNS therapy. In these patients, the median time from randomization to second disease progression or death was for TTC 15.9 vs. 9.7 months for placebo (HR 0.33; 95% CI 0.11–0.02).

Other Treatments

The combination of bevacizumab, etoposide and cisplatin (BEEP) was studied in 1 study of 23 patients (54.3% with an ECOG PS of 2 or 3), all of whom had progressive disease after prior whole brain radiotherapy (WBRT) (Table 1) [30]. It was the only study in this meta-analysis in which treatment did not consist of a HER2 targeting agent. The hypothesis was that a window period between bevacizumab and cytotoxic agents might enhance drug delivery to tumor tissue through bevacizumab-induced vascular normalization in patients with mBC and BM [30]. Patients in this study achieved an ORR of 69.6% (Figure 3), mPFS of 7.7 months (95% CI 6.6–8.8) and mOS of 11.8 months (95% CI 7.0–16.6). However, there is a serious risk of bias in outcome measurement due to the use of volumetric response criteria instead of RECIST or RANO, while part of the volumetric response might be due to effective treatment of radionecrosis by bevacizumab instead of representing effective anticancer treatment. Moreover, the study was constrained to the use of contrast-enhanced images for efficacy assessment instead of MRI T2/FLAIRE images because of post-WBRT diffuse white matter changes.

The effect of everolimus, a mTOR inhibitor was investigated in two studies, combinedly including 51 patients (Table 4). Previous results showed that hyperactivation of the PI3K/mTOR pathway during treatment with trastuzumab correlated with poor OS and increased risk of BM [86]. Thus, inhibition of the PI3K/mTOR pathway, combined with HER2-directed therapy, may yield more sustained responses for patients with HER2+ mBC and BM. Swearingen et al. combined everolimus with vinorelbine in 32 patients (97% prior local treatment for BM) and demonstrated an ORR of 4% (Figure 3), a mPFS of 3.9 months (95% CI 2.3–5.0) and a mOS of 12.1 months (95% CI 6.8–12.4); this schedule was deemed ineffective [35]. Hurvitz et al. combined everolimus with LC and included 19 patients (63% prior local treatment for BM) with less extracranial disease compared to the Swearingen study (42% vs. 66%); they reported an ORR of 28% (Figure 3), a mPFS of 6.2 months and a mOS of 24.2 months [36]. Accrual goals were not met. Importantly, 73% of patients were not pretreated with LC, thus the ORR of 28% could represent the ORR of LC instead of an additive effect of everolimus.

Discussion

We present a complete overview of systemic treatment options in HER2+ mBC with BM. Interpretation of the meta-analysis is limited by the high level of heterogeneity and risk of bias of the available studies. Best quality data and/or highest ORR in ≥2nd line were demonstrated in studies evaluating T-Dxd and tucatinib. We should take into account that patients in T-DM1, pyrotinib and LC studies received fewer prior treatments compared to T-Dxd and tucatinib. Concomitant local therapy, comedication, active/stable BM and ECOG status differed. Comparisons are mostly made based on CNS ORR, but not only BM response influences prognosis. The systemic disease status is also relevant and quite different in patients included in the different studies. Based on the CLEOPATRA study data, the combination of TP and a taxane is considered standard first line therapy. In the CLEOPATRA study, median time to CNS PFS was delayed (15 vs. 11.9 months; HR 058; $p = 0.0049$). However, its efficacy for patients with baseline BM was only described in combination with local therapy in the RePer study and by Bergen et al. [43,44]. In later lines of therapy, reintroducing trastuzumab at a higher dosage was not effective.
Of all systemic therapies, T-DXd showed the highest pooled ORR (64%) in patients with HER2+ mBC and stable BM in ≥2nd line. Importantly, this effect was shown in a heavily pretreated population. Ongoing prospective studies on T-DXd will provide us with more data on its effect in patients with stable BM in the DESTINY Breast12 trial (ClinicalTrials.gov identifier: NCT04739761) and with active BM in the TUXEDO-1 trial (ClinicalTrials.gov identifier: NCT04752059), which reported promising first results [40]. In ≥2 line, TTC achieved a high ORR of 47% in the HER2CLIMB study; importantly this is the only well performed double blind randomized trial, demonstrating a mOS benefit for patients with BM. TTC is the only therapy studied for the treatment of active BM. At this moment, no comparative data between T-DXd and tucatinib are available. A direct comparison using currently available data is difficult, as in the HER2CLIMB trial no previous treatment with TKIs was allowed, in contrast to the Destiny Breast03 trial. The combination of tucatinib and T-DXd is currently being studied in the HER2CLIMB-4 study (ClinicalTrials.gov identifier: NCT04539938).

For Asian patients, pyrotinib is another ≥2nd line treatment option, demonstrating a pooled ORR of 43% and pooled mPFS of 10 months. However, most studies used retrospectively acquired data. Patients had received only a median of 1 prior treatment line, and almost no prior T-DM1 or pertuzumab. Moreover, ORR was mainly high in the radiotherapy-naïve group. Pyrotinib was directly compared to LC and capecitabine monotherapy in the Phoebe and Phenix trials, respectively. In these trials, pyrotinib demonstrated a superior efficacy in Asian patients in general. [71,84]. However, the number of patients with baseline BM in the Phoebe and Phenix trials was small, and occurrence of BM was comparable between pyrotinib and control arm (2%) [84], so more prospective data are needed regarding the efficacy of pyrotinib in patients with BM.

When opting for T-DM1 or LC in ≥3rd line, a comparable pooled ORR of 28% was found, although pooled mPFS was slightly longer for T-DM1 than for LC. Based on a direct comparison of T-DM1 and LC in the randomized phase 3 Emilia study, T-DM1 outperformed LC in terms of mOS in the selected group with baseline BM (26.8 vs. 12.9 months, p = 0.008) [45,80]. T-DM1 treated patients without baseline BM had a higher occurrence of BM over the course of their disease vs. LC treated patients (3.8% vs. 0.2%; p = NS). Regarding LC, the CEREBEL trial compared LC to trastuzumab and capecitabine (TC) in 501 patients with HER2+ mBC, and LC demonstrated a lower incidence of BM as first site of relapse than TC (3% vs. 5%; p = NS) [87].

NC demonstrated an ORR of 29% [34] and 49% [33], in two studies. When opting for treatment with either NC or LC, the randomized phase 3 NALA study can provide guidance, as it directly compared both therapies in 101 patients with HER2+ mBC and BM. Only a non-significant moderately improved mPFS was demonstrated for NC over LC and a comparable ORR was found [34]. However, patients treated with NC required significantly less interventions for BM (22.8% for NC vs. 29.2% for LC, p = 0.043), providing a hint of improved intracranial efficacy for NC over LC [88]. For NC, both cost and drug availability might be an issue as well as adverse events, as NC leads to diarrhea more frequently than LC [88].

Studies investigating everolimus, lapatinib monotherapy, cabozantinib or afatinib did not demonstrate a clinically relevant effect and/or included a low number of patients and should therefore currently not be considered for treating patients with HER2+ mBC with BM.
An important observation is that although the BBB is known to reduce efficacy of systemic treatments especially in preclinical models, with current TKIs and ADCs, we now have evidence of effective intracranial treatments for patients with BM, although mOS remains shorter than in patients without BM. Choices in sequential therapies can be made weighing ORR, mPFS, mOS, adverse events, availability and cost.
Although the best order is not known, T-DXd and TTC are the most effective systemic treatment options to date in patients with HER2+ mBC and BM. In clinical practice, we would currently recommend T-DXd or TTC for second line treatment, realizing that both may become available for first line therapy in the near future. In case these drugs are not available, we would suggest pyrotinib for Asian patients. No further recommendations can be made due to low patient numbers and heterogeneity of the included studies.
This review provides an overview and insight in interpreting the efficacy of drugs in patients with HER2+ mBC and BM, acknowledging the heterogeneity and sometimes low quality of included studies. Preferably, future research will comprise of randomized controlled trials, including patients with active and/or inactive BM. Based on current knowledge, we would hypothesize that the most effective first line treatments in the future will consist of ADC's. Importantly, in the current treatment landscape, patients receiving multiple lines of anti-HER2 therapy, administered after BM diagnosis, have a significantly improved mOS [89].

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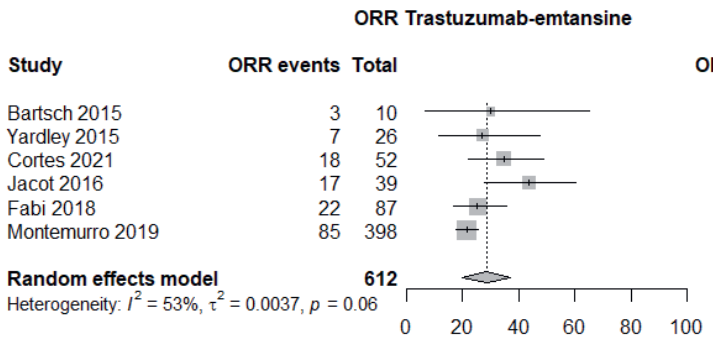
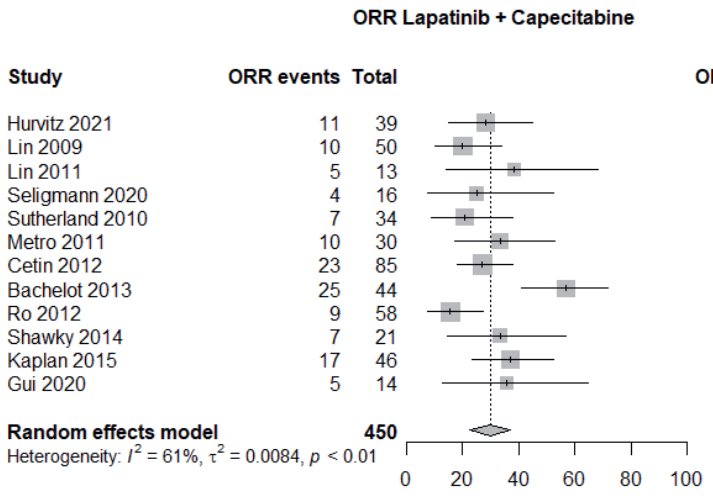
Supplementary Figure 1: Sensitivity analysis (excluding abstract-only articles) pooled ORR (%) meta-analysis
A. Trastuzumab-emtansine (T-DM1); B. Lapatinib + capecitabine (LC)

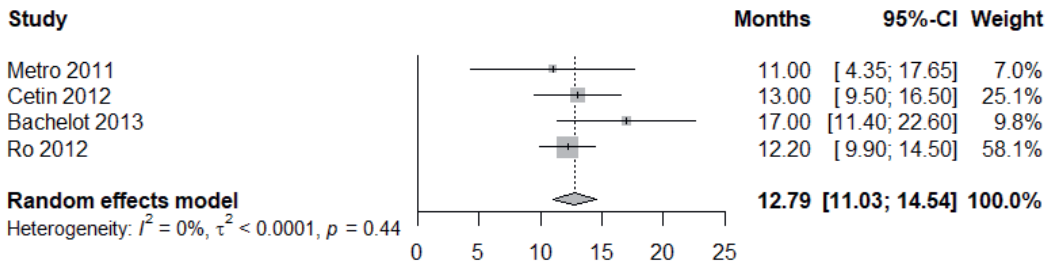
A



B



Supplementary Figure 2: Sensitivity analysis (excluding abstract-only articles) pooled mOS (months) meta-analysis: Lapatinib + capecitabine (LC)



Supplementary File 1 search strategy

METHODS SECTION
A systematic search was performed in the databases: PubMed, Embase.com, Clarivate Analytics/Web of Science Core Collection and the Wiley/Cochrane Library. The timeframe within the databases was from inception to 19th January 2022 and conducted by GBL and IW. The search included keywords and free text terms for (synonyms of) 'breast neoplasm' combined with (synonyms of) ‘erythroblastic oncogene B’ combined with (synonyms of) ‘brain metastasis’. Reviews, animal studies, comments, letters, editorials, qualitative studies, case reports and case series were excluded from the search. A full overview of the search terms per database can be found in the supplementary information (see appendix 1). No limitations on date or language were applied in the search.

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Appendix 1

Table 1: Search strategy in PubMed

Search	Query	Results
#9	#8 NOT ("Case Reports"[Publication Type] OR "case report*"[tiab] OR "case stud*"[tiab] OR "case histor*"[tiab] OR "case serie*"[tiab])	556
#8	#7 NOT ("Qualitative Research"[Mesh] OR "Focus Groups"[Mesh] OR "Interview" [Publication Type] OR "Interviews as Topic"[Mesh] OR "Narration"[Mesh] OR "Personal Narratives as Topic"[Mesh] OR "Observational Studies as Topic"[Mesh] OR "Observational Study"[Publication Type] OR "Tape Recording"[Mesh] OR "Grounded Theory"[Mesh] OR "thematic analys*"[tiab] OR "content analys*"[tiab] OR "focus group*"[tiab] OR "ethnograph*"[tiab] OR "ethnograf*"[tiab] OR "etnograf*"[tiab] OR "field stud*"[tiab] OR "phenomenolog*"[tiab] OR "narration*"[tiab] OR "narrative"[tiab] "case stud*"[tiab] OR "qualitative stud*"[tiab] OR "qualitative analys*"[tiab] OR "qualitative research*"[tiab] OR "qualitative method*"[tiab] OR "multimethodolog*"[tiab] OR "mixed method*"[tiab] OR "observation*"[tiab] OR "grounded theor*"[tiab] OR "audio recording*"[tiab] OR "tape recording*"[tiab] OR "audiotape*"[tiab] OR (("semi-structured"[tiab] OR "semistructured"[tiab] OR "unstructured"[tiab] OR "informal"[tiab] OR "in-depth"[tiab] OR "indepth"[tiab] OR "face-to-face"[tiab] OR "structured"[tiab] OR "guide*"[tiab]) AND ("interview*"[tiab] OR "discussion*"[tiab] OR "questionnaire*"[tiab])))	629
#7	#6 NOT ("Comment" [Publication Type] OR "Letter" [Publication Type] OR "Editorial" [Publication Type])	650
#6	#5 NOT ("Animals"[Mesh] NOT "Humans"[Mesh])	683
#5	#4 NOT ("systematic review"[tiab] OR "systematic literature review*"[tiab] OR "review*"[tiab] OR "Review"[Publication Type] OR "Meta-Analysis as Topic"[Mesh] OR "meta-analysis"[tiab] OR "Meta-Analysis"[Publication Type])	698
#4	#1 AND #2 AND #3	976

Search	Query	Results
#3	"central nervous system metasta*"[tiab] OR "CNS metasta*"[tiab] OR "brain metasta*"[tiab] OR "metastasis to the brain*"[tiab] OR "metastasized to the brain"[tiab] OR "metastasised to the brain"[tiab] OR "metastasis to the CNS*"[tiab] OR "metastasized to the CNS"[tiab] OR "metastasised to the CNS"[tiab] OR "metastasis to the central nervous system*"[tiab] OR "metastasized to the central nervous system*"[tiab] OR "metastasised to the central nervous system*"[tiab]	16,228
#2	"Genes, erbB-2"[Mesh] OR "ERBB2 protein, human"[Supplementary Concept] OR "erythroblastic oncogene B"[tiab] OR "erbB2*"[tiab] OR "erbB 2*"[tiab] OR "Erbb2"[tiab] OR "Erbb 2"[tiab] OR "neugene*"[tiab] OR "neu gene*"[tiab] OR "proto-oncogene Neu*"[tiab] OR "human epidermal growth factor receptor 2*"[tiab] OR "HER2*"[tiab] OR "HER 2*"[tiab] OR "HER2/neu"[tiab] OR "CD340"[tiab] OR "CD 340"[tiab]	50,345
#1	"Breast Neoplasms"[Mesh] OR (("breast*"[tiab] OR "mamma*"[tiab]) AND ("cancer*"[tiab] OR "carcinom*"[tiab] OR "malignan*"[tiab] OR "metasta*"[tiab] OR "neoplas*"[tiab] OR "tumor*"[tiab] OR "tumour*"[tiab]))	504,112

Table 2: Search strategy in Embase.com

Search	Query	Results
#9	#8 NOT ('case report'/exp OR ("case report*" OR "case stud*" OR "case histor*" OR "case serie*"):ti,ab,kw)	**1,092**
#8	#7 NOT ('qualitative research'/exp OR 'interview'/exp OR 'narrative'/exp OR 'observational study'/exp OR 'recording'/exp OR 'grounded theory'/exp OR ('thematic analys*' OR 'content analys*' OR 'focus group*' OR 'ethnograph*' OR 'ethnograf*' OR 'etnograf*' OR 'field stud*' OR 'phenomenolog*' OR 'narration*' OR 'narrative' 'case stud*' OR 'qualitative stud*' OR 'qualitative analys*' OR 'qualitative research*' OR 'qualitative method*' OR 'multimethodolog*' OR 'mixed method*' OR 'observation*' OR 'grounded theor*' OR 'audio recording*' OR 'tape recording*' OR 'audiotape*'):ti,ab,kw OR (('semi-structured' OR 'semistructured' OR 'unstructured' OR 'informal' OR 'in-depth' OR 'indepth' OR 'face-to-face' OR 'structured' OR 'guide*'):ti,ab,kw AND ('interview*' OR 'discussion*' OR 'questionnaire*'):ti,ab,kw))	**1,248**
#7	#6 NOT ('conference abstract'/it OR 'conference review'/it OR 'editorial'/it OR 'erratum'/it OR 'letter'/it OR 'note'/it OR 'short survey'/it))	**1,304**
#6	#5 NOT ([animals]/lim NOT [humans]/lim)	**2,493**
#5	#4 NOT ('systematic review'/exp OR 'meta analysis'/exp OR ('systematic literature review' OR 'systematic review*' OR 'meta-analys*' OR 'review*'):ab,ti,kw)	**2,605**
#4	#1 AND #2 AND #3	**3,352**
#3	'central nervous system metastasis'/exp OR ('central nervous system metasta*' OR 'CNS metasta*' OR 'brain metasta*' OR 'metastasis to the brain*' OR 'metastasized to the brain' OR 'metastasised to the brain' OR 'metastasis to the CNS*' OR 'metastasized to the CNS' OR 'metastasised to the CNS' OR	**50,534**

Search	Query	Results
	‘metastasis to the central nervous system*’ OR ‘metastasized to the central nervous system*’ OR ‘metastasised to the central nervous system*’):ti,ab,kw	
#2	'oncogene neu'/exp OR 'epidermal growth factor receptor 2'/exp OR 'human epidermal growth factor receptor 2 positive breast cancer'/exp OR (‘erythroblastic oncogene B’ OR ‘erbB2*’ OR ‘erbB 2*’ OR ‘Erbb2’ OR ‘Erbb 2’ OR ‘neugene*’ OR ‘neu gene*’ OR ‘proto-oncogene Neu*’ OR ‘human epidermal growth factor receptor 2*’ OR ‘HER2*’ OR ‘HER 2*’ OR ‘HER2/neu’ OR ‘CD340’ OR ‘CD 340’):ti,ab,kw	**108,102**
#1	'breast tumor'/de OR 'breast cancer'/exp OR 'experimental mammary neoplasm'/exp OR ((‘breast*’ OR ‘mamma*’):ti,ab,kw AND (‘cancer*’ OR ‘carcinom*’ OR ‘malignan*’ OR ‘metasta*’ OR ‘neoplas*’ OR ‘tumor*’ OR ‘tumour*’):ti,ab,kw)	**760,299**

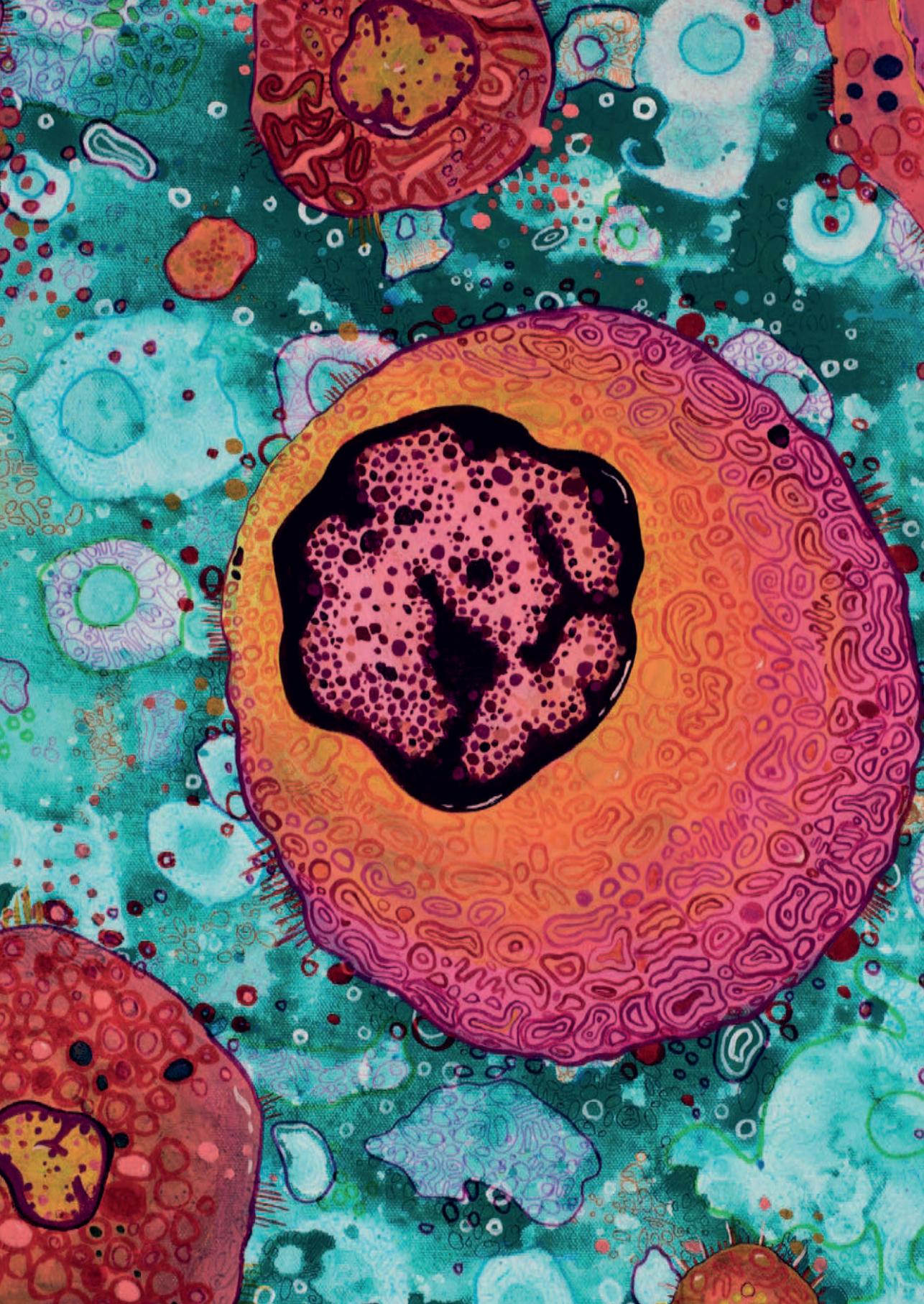
Table 3: Search strategy in Clarivate Analytics/Web of Science Core Collection

Search	Query	Results
#7	#6 NOT TS=("case report*" OR "case stud*" OR "case histor*" OR "case serie*")	**1,031**
#6	#5 NOT TS=("thematic analys*" OR "content analys*" OR "focus group*" OR "ethnograph*" OR "ethnograf*" OR "etnograf*" OR "field stud*" OR "phenomenolog*" OR "narration*" OR "narrative" "case stud*" OR "qualitative stud*" OR "qualitative analys*" OR "qualitative research*" OR "qualitative method*" OR "multimethodolog*" OR "mixed method*" OR "observation*" OR "grounded theor*" OR "audio recording*" OR "tape recording*" OR "audiotape*" OR (("semi-structured" OR "semistructured" OR "unstructured" OR "informal" OR "in-depth" OR "indepth" OR "face-to-face" OR "structured" OR "guide*") AND ("interview*" OR "discussion*" OR "questionnaire*")))	**1,063**
#5	#4 NOT TS=("systematic review" OR "systematic literature review*" OR "review*" OR "meta-analysis")	**1,090**
#4	#1 AND #2 AND #3	**1,379**
#3	TS=("central nervous system metasta*" OR "CNS metasta*" OR "brain metasta*" OR "metastasis to the brain*" OR "metastasized to the brain" OR "metastasised to the brain" OR "metastasis to the CNS*" OR "metastasized to the CNS" OR "metastasised to the CNS" OR "metastasis to the central nervous system*" OR "metastasized to the central nervous system*" OR "metastasised to the central nervous system*")	**22,331**
#2	TS=("erythroblastic oncogene B" OR "erbB2*" OR "erbB 2*" OR "Erbb2" OR "Erbb 2" OR "neugene*" OR "neu gene*" OR "proto-oncogene Neu*" OR "human epidermal growth factor receptor 2*" OR "HER2*" OR "HER 2*" OR "HER2/neu" OR "CD340" OR "CD 340")	**64,660**
#1	TS=(("breast*" OR "mamma*") AND ("cancer*" OR "carcinom*" OR "malignan*" OR "metasta*" OR "neoplas*" OR "tumor*" OR "tumour*")))	**725,440**

Table 4: Search strategy in Wiley/Cochrane Library

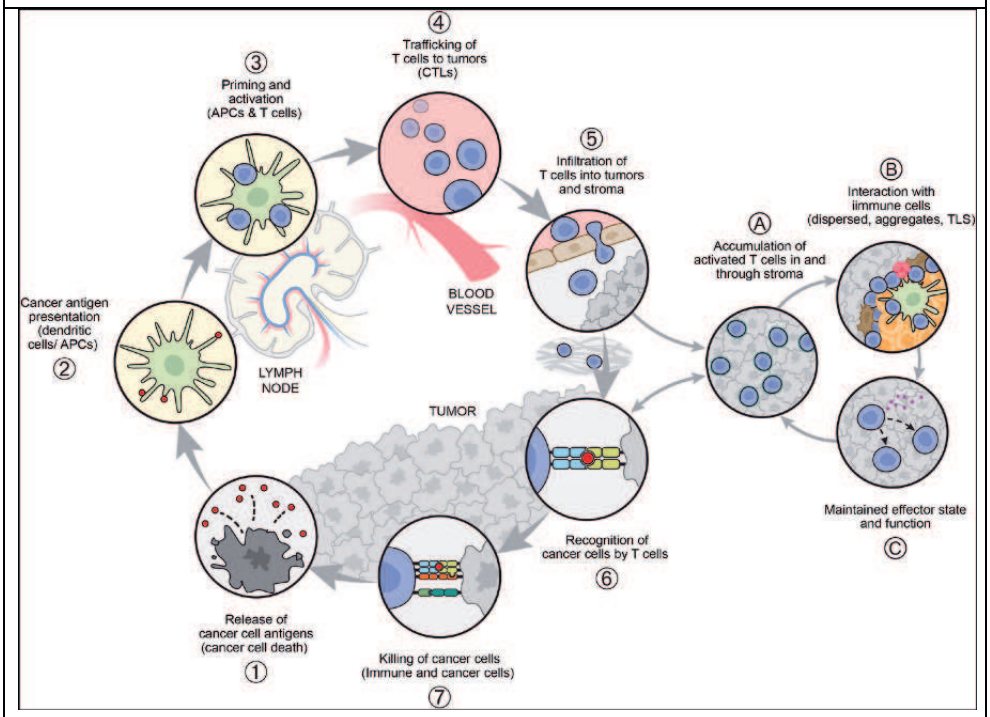
Search	Query	Results
#4	#1 AND #2 AND #3	**1**
#3	(“central nervous system metasta*” OR “CNS metasta*” OR “brain metasta*” OR “metastasis to the brain*” OR “metastasized to the brain” OR “metastasised to the brain” OR “metastasis to the CNS*” OR “metastasized to the CNS” OR “metastasised to the CNS” OR “metastasis to the central nervous system*” OR “metastasized to the central nervous system*” OR “metastasised to the central nervous system*”):ti,ab,kw	**14**
#2	(“erythroblastic oncogene B” OR “erbB2*” OR “erbB 2*” OR “Erbb2” OR “Erbb 2” OR “neugene*” OR “neu gene*” OR “proto-oncogene Neu*” OR “human epidermal growth factor receptor 2*” OR “HER2*” OR “HER 2*” OR “HER2/neu” OR “CD340” OR “CD 340”):ti,ab,kw	**7,240**
#1	((“breast*” OR “mamma*”):ti,ab,kw AND (“cancer*” OR “carcinom*” OR “malignan*” OR “metasta*” OR “neoplas*” OR “tumor*” OR “tumour*”):ti,ab,kw)	**39,812**

Chapter 6 Summarizing Discussion and future perspectives



The main focus of the research described in this thesis is on immunomodulation in cancer therapy. A lot has changed in the landscape of cancer treatment since the National Cancer Act of 1971 when “the war on cancer” was declared by United States president Richard Nixon [1]. Cancer therapy modalities comprise, amongst others, surgery, radiation therapy, chemotherapy, hormonal therapy, targeted therapy, immunotherapy, and antibody-drug conjugates (ADC). Despite the advancements in these therapeutic modalities, there remains a significant need for further research and improvement, as a considerable number of patients continue to succumb to their disease following a cancer diagnosis. In the immunotherapeutic era of treating cancer patients, various means of enhancing the functionality of the immune system have been studied. The cancer-immunity cycle (CIC) provides a framework to understand the series of events that generate anti-cancer immune responses (Figure 1) [2].

Figure 1. The cancer-immunity cycle (adapted from [2])



The CIC can be divided into sequential critical steps, beginning with;
Step 1- release of cancer antigens;
Step 2- cancer antigen presentation;
Step 3- priming and activation in secondary lymphoid organs;
Step 4- trafficking of T cells to tumors;
Step 5- infiltration of T cells into tumors;
Step 6- recognition of cancer cells by T cells;
Step 7- killing of cancer cells [3].

The CIC can be impaired at any step and immunomodulation is aimed at overcoming blockades and reactivating the CIC to facilitate tumor recognition and eradication. In this thesis we described different therapeutic strategies in breast cancer (BC) and metastatic renal cell cancer (mRCC) and here we will discuss their effect within the context of the CIC.
Chemotherapy, radiation therapy, ADC, oncolytic viruses and targeted therapy can induce apoptosis of tumor cells , leading to an increased release of cancer antigen and thereby providing the first step in the CIC to activate the immune system. The ADC Trastuzumab-deruxtecan (T-Dxd) specifically binds to Her2+ (cancer) cells, leading to a targeted effect of the cytostatic deruxtecan component, thus leading to the selective death of targeted tumor cells and the subsequent release of cancer antigens, along with damage associated molecular patterns (DAMPs) that can activate DC and so trigger an antitumor T-cell response (i.e. immunogenic cell death). In the systematic review and meta-analysis in **Chapter 5** T-Dxd appeared to be the most effective treatment modality, leading to highest objective response rates (ORR) in patients with Her2+ metastatic BC (mBC) and brain metastasis (BM), although this meta-analysis should be interpreted with caution due to heterogeneity of included studies and a related serious risk of bias. BM are challenging for the development of effective anticancer therapies, as conventional chemotherapy is known to be less effective in this sanctuary site, due to the blood-brain-barrier (BBB). Moreover BM have mechanisms to avoid immune detection: they alter T–cell ligand and co-stimulatory molecule expression, activate and suppress microglia, activate immunosuppressive tumor associated macrophages (TAM), secrete anti-inflammatory cytokines, downregulate proteins needed for antigen presentation, and upregulate angiogenic factor expression [4]. Nevertheless, due to its leaky nature in cancer, the BBB can be crossed by T cells and antibodies, evidenced by tumor responses observed upon immune checkpoint blockade. T-Dxd is currently studied more extensively in patients with Her2+ mBC and BM, and we are awaiting the results of the DESTINY Breast12 trial, including patients with stable BM (ClinicalTrials.gov identifier: NCT04739761) [5] and the HER2CLIMB-4 study, investigating the combination of tucatinib and T-Dxd in patients with and without BM (ClinicalTrials.gov identifier: NCT04539938) [6].

Since the publication of our meta-analysis, the updated results of the Tuxedo 1 trial were published, in which patients with Her2+ mBC and active BM were studied. This study demonstrated an ORR of 83.3% in the first 10 patients (included in the meta-analyses), the updated analysis of 15 included patients demonstrated a slightly lower ORR of 73.3% [7]. However, this will not influence the results of the meta-analysis significantly and we still maintain that T-Dxd is the most effective drug studied in patients with Her2+ mBC and BM.
As mentioned before, one explanation for the immunological effects of the ADC T-Dxd could be the release of cancer antigens in an immunogenic fashion. Another factor might be that BC cells exhibiting high Her2 expression have down-regulation of MHC class I expression, inhibiting CD8+ T cell recognition [8]. Blockade of the Her2 receptor by T-Dxd could restore MHC class 1 expression and therefor promote T cell recognition (Step 6 CIC, recognition of cancer cells by T cells). Also, the trastuzumab compound of T-Dxd is known to have an effect through antibody-dependent cell-mediated cytotoxicity (ADCC) [9], implying a role for T-Dxd in step 7 of the CIC (killing of cancer cells).
Some ADCs conjugated with tubulysin or pyrrolobenzodiazepine dimer showed immune-activating effects and benefit in combination with checkpoint-inhibitors (CPI) in immunocompetent BC mouse models [10]. In these mouse models, it was shown that the administration of T-Dxd led to both increased tumor-infiltrating activated dendritic cells (DC) and $CD8^+$ T cells as well as enhanced PD-L1 and MHC class I expression on tumor cells [10]. T-Dxd was combined with respectively an anti-PD1-antibody or a CTLA-4 antibody in these mouse models and both combinations were deemed more effective than either monotherapy [10, 11]. Unfortunately, in an open-label, multicenter, phase 1b study the addition of nivolumab to T-Dxd showed no discernable benefit in the late-line setting of 48 patients with mBC (Her2+ n=32; Her2 low n=16) [12]. Besides these hypotheses and mouse studies, little is actually known about the effects of T-Dxd on the immune response, as most clinical trials lack immune monitoring programs to obtain immunological translational data. It would be very interesting to further study the immunological effects of T-Dxd in patients with mBC and explore strategies to strengthen the immune system, exemplified by promising effects of ADC combined with CPI in urothelial cancer [13].

ADCs are an ideal option for combination therapy as they are able to selectively target cancer cells (expressing e.g. Her2) and spare normal cells, the conjugated antibody's considerable size and hydrophilic character significantly mitigate nonspecific absorption, thereby augmenting the specificity and safety of ADCs [14].
To yield an anticancer T cell response, DC should present antigens via MHC class I and MHC class II to T cells. Conventional DC type 1 (cDC1) are the most important CD8+ T cell initiators through MHC class I, while cDC2 are more prone to stimulate CD4+ T cells through MHC class II [15]. DC appear to be far more complex than just antigen-presenters and initiators of a T cell response. DC also serve as the most important source of PD-L1 and are thereby responsible for supporting and maintaining T cell responses upon PD-(L)1 blockade [2]. Immune desert tumors do not only lack T cells in the tumor microenvironment (TME), but also lack DC. The role of DC in the TME is not limited to the transfer of antigens from tumor to tumor draining lymph nodes (TDLN), but also to ensure the attraction, activation and expansion of antigen-specific T cells in the tumor itself (step 3 of the CIC, priming and activation in secondary lymphoid organs and also substeps 5A-C, priming and activation of the local TME) [2]. In the phase 3 Spinoza trial, described in **Chapter 4**, we tried to boost DCs to create a more robust immune response in the treatment of patients with locally advanced BC (LABC) by the addition of growth factor GM-CSF to neoadjuvant chemotherapy (NAC). BC is generally considered immune excluded or an immune desert and could therefore benefit from activation of DCs, especially because LABC comprise a poor prognostic group of patients with a high risk of micrometastasis due to their large tumor size, invasion of nearby tissues and/or spread to regional lymph nodes. Indeed we showed an enhanced systemic DC differentiation in patients with LABC upon NAC, supported by either GM-CSF or G-CSF. Notably, GM-CSF appeared to be more potent in stimulating maturation of DC in TDLN. GM-CSF supported NAC could thus possibly create a more robust anti-tumor immune response than current G-CSF based NAC strategies. Importantly, systemic concentrations of GM-CSF and duration of exposure to GM-CSF are important to take into account when designing future studies, as these factors might influence GM-CSF induced immunologic effects [16, 17].

The most successful and revolutionizing immunotherapy approach until now consist of the (approved) CPI targeting anti–programmed death 1 (PD-1), anti–programmed death ligand (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and more recently LAG-3 [18]. These CPI block checkpoint proteins, such as PD-L1 on tumor cells and PD-1 on T cells, allowing the T cells to kill tumor cells. CPI have their effect in step 3 (priming and activation in secondary lymphoid organs) and step 7 (killing of cancer cells) of the CIC. In this thesis, mRCC was studied at a time when CPI were not yet approved for this indication. Sunitinib, a multi-targeted tyrosine kinase inhibitor (TKI) had been the standard first-line treatment option [19], followed by everolimus, a mTOR inhibitor, as a standard second-line treatment option [20]. Since 2018 various options have been added to the therapeutic arsenal in mRCC patients, including (a) the combination of the anti-PD1 CPI nivolumab plus the anti-CTLA-4 CPI ipilimumab [21], (b) the anti-PD-1 CPI pembrolizumab plus axitinib [22], (c) nivolumab plus cabozantinib [23] and (d) pembrolizumab plus lenvatinib [24]. Choosing the best therapeutic strategy for mRCC has become more difficult with all these options, balancing choices between effect, time to response, toxicity, and costs. Insight into the rationale for the different treatment modalities could aid in decision making. Unfortunately most of the large clinical trials lack extensive immunomonitoring data. Many immunological cell subsets express CTLA-4 and/or PD-1 and/or PD-L1 and immunomonitoring programs could aid in the detection of biomarkers or create a biomarker profile for patients with an expected good response to CPI.

In the past era when there were fewer effective treatment options for mRCC, we sought to improve the at that time standard second-line treatment with everolimus. Everolimus was demonstrated to affect multiple immune cell subsets, especially Tregs, and altogether tip the balance in favor of immunosuppression [25]. Tregs can suppress the activation, expansion, and function of other T cells. They express CD4, CD25, and the immune suppression-related transcription factor Forkhead box P3 (Foxp3) and are important mediators of immunosuppressive responses, thereby preventing auto-immunity. An immunosuppressive environment created by Tregs can provide an inadequate antitumor response, creating the ideal situation for cancer cells to develop and grow.

Tumor cells or TAMs produce chemokines (e.g. C motif chemokine ligand 22 (CCL22)) to escape anti-tumor immunity by attracting Tregs, e.g. through C-C chemokine receptor 4 (CCR4) [26, 27]. The amount of circulating and (peri)tumoral Tregs are associated with worse survival in cancer patients [28]. A strategy in mRCC to steer towards a more immunostimulatory combination therapy by counteracting the everolimus-induced Treg increase, preferably by actual depletion of Tregs, was (and is) considered an approach worthy of exploration. Depletion of Tregs can be achieved by various ways. From preclinical studies there is evidence that blocking IL-2 or targeting CD25, the cell surface expressed α chain of the IL-2 receptor, and blocking the CCR4 receptor or its ligand CCL22, are effective strategies to reduce the amount and functionality of Tregs [26, 29-31]. Therefore clinical phase 1-2 studies aimed at depleting Tregs have included the anti-CCR4 monoclonal antibody (mAb) Mogalizumab [32], (variants of) the anti-CD25 mAb Daclizumab [33-35] and ONTAK, an IL2/ diphtheria toxin conjugate [36-41]. Other means of depleting Tregs could be achieved by the administration of the glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) antibody TRX518, thereby neutralizing GITR, which is preferentially expressed on Tregs [42] or by downregulating Foxp3 gene expression by the histone deacetylase (HDAC) inhibitor entinostat [43]. Another trial aimed at broadly inhibiting inflammation by blocking Cox-2, thereby reducing CCL22 levels and subsequently impairing Treg recruitment, through the administration of NSAID M2000 [44]. Since the introduction of the anti-CTLA-4 CPI, there have been reports indicating their role in depleting Tregs, possibly through ADCC, although results are not uniform [45, 46] Another approach to deplete Tregs consists of the administration of metronomic cyclophosphamide, an alkylating agent of the nitrogen mustard type, which can selectively deplete Tregs (and not T-helper cells or cytotoxic T cells), most probably by causing a DNA repair defect [47]. Various phase 1-2 clinical studies have been done with cyclophosphamide in different doses and schedules as a modulator to deplete Tregs [48-55]. As there was controversy on the optimal Treg depletion dose and schedule of cyclophosphamide, we performed a phase 1 clinical trial in which everolimus was combined with several schedules of metronomic oral cyclophosphamide in patients with mRCC, the results of which are described in **Chapter 2.**

We demonstrated that selective and significant Treg depletion in peripheral blood could be achieved when patients with mRCC were treated with a standard once daily therapeutic dose of 10mg everolimus combined with 50mg cyclophosphamide once daily. After four weeks of combination treatment absolute Treg numbers and Treg percentages decreased. This combination of once daily oral 50mg cyclophosphamide and 10mg everolimus was selected for the phase 2 part of the trial. Several adverse events (AE) were recorded, and the most common side effects were fatigue, anorexia, rash, cough, mucositis, nausea, anemia and hypercholesterolemia. The overall incidence of these AEs was in the same range as that of everolimus monotherapy [20].
In **Chapter 3** the phase 2 clinical trial evaluating the combination of everolimus and metronomic cyclophophamide is outlined. The trial aimed at improving progression free survival (PFS) of patients with mRCC by the addition of cyclophosphamide to everolimus, compared to everolimus monotherapy. Treg modulating effects of metronomic cyclophosphamide were comparable to those observed in the phase 1 part of the study, however the phase 2 trial was abrogated at the predefined interim analysis after inclusion of 24 patients, since the PFS did not improve from 50% to the pre-set 70% at 4 months. The immunomodulatory effects of the combination of metronomic cyclophosphamide and everolimus did not translate into an altered clinical outcome, possible due to the fact that the Tregs that persisted in the peripheral blood were proliferating and had strong inhibitory functions, implicating some sort of recruitment of these cells, perhaps as a feedback mechanism. This might in part explain why the observed (temporary) reduction in Treg levels was not enough to achieve effective and lasting tumor immunity and therefore could not be translated into an improved clinical outcome. Despite the termination of the phase 2 part of the trial, we did obtain relevant information from the comprehensive immunomonitoring data that may be taken into account in the design of future immunotherapeutic approaches that incorporate or are based on mTOR inhibitors. For example. mTOR inhibition could be an interesting lead in overcoming resistance in BRAF-mutated melanoma by inhibiting the PI3K/AKT/mTOR pathway and development of Tregs could then be an interesting prognostic key for renewed resistance [56]. Furthermore, the immunomodulatory effects of treatment with cyclophosphamide as described in this thesis, may be of additional value, when combined with CPI, simultaneously or in a sequential manner.

We saw a decrease in immunoregulatory natural killer (NK) cells and an increase in cytotoxic NK cell by the addition of cyclophosphamide, moreover cyclophosphamide induced a reduction in myeloid-derived suppressor cells (MDSC). It has been suggested that the failure of CPI could be partially attributed to the persistent suppressive role of MDSC or the variability in activation of NK cells [57, 58]; both could be counteracted by the addition of cyclophosphamide.
Nowadays the use of everolimus monotherapy for mRCC has been mostly abandoned, due to the introduction of more effective therapeutic alternatives. Everolimus is still in use in combination with lenvatinib , showing increased efficacy in a phase 2 trial [59]. A possible mechanism underpinning the acquired resistance to everolimus might be that inhibiting mTORC1 by everolimus results in mTORC2-dependent activation of AKT with the STAT3 and ERK pathways and upregulation of hypoxia-inducible transcription factors (HIF) [60]. When lenvatinib was combined with everolimus, lenvatinib could partly counteract one of these resistance mechanism of everolimus by blocking VEGF and thereby downregulating HIF.

Concluding remarks

The different therapeutic strategies described for BC and mRCC in this thesis have their predicted impact in one or more steps of the CIC. Understanding the possible effects of new cancer strategies on an immunological level is key in moving forward towards more effective therapies in all cancer patients. Especially tumors that exhibit immune excluded phenotypes are a major challenge in the development of these immunotherapeutic strategies. We believe that future early-phase as well as phase 3 randomized trials in cancer therapy should incorporate translational analyses as new immunotherapeutic concepts could be more effective based on the knowledge of different immunotypes and the CIC-framework. Translational research might help us in understanding the differential immunological effects of treatment strategies and finding the right (dose-adjusted) combination therapy to treat more types of cancer in all different immunotype patients.

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Part Three: Appendices

Dutch summary (Nederlandse samenvatting)

Acknowledgements (Dankwoord)

About the Author (Curriculum Vitae)

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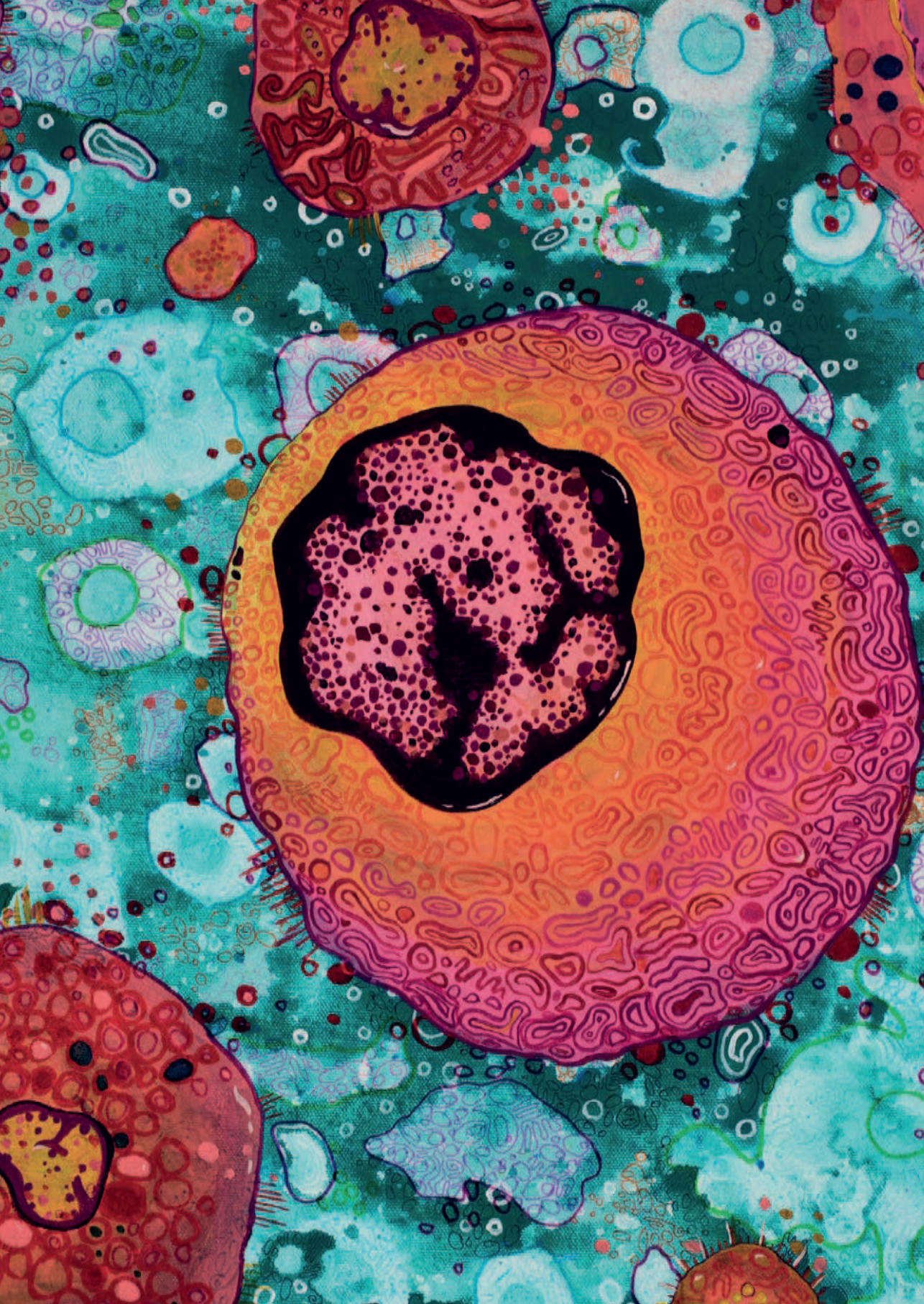
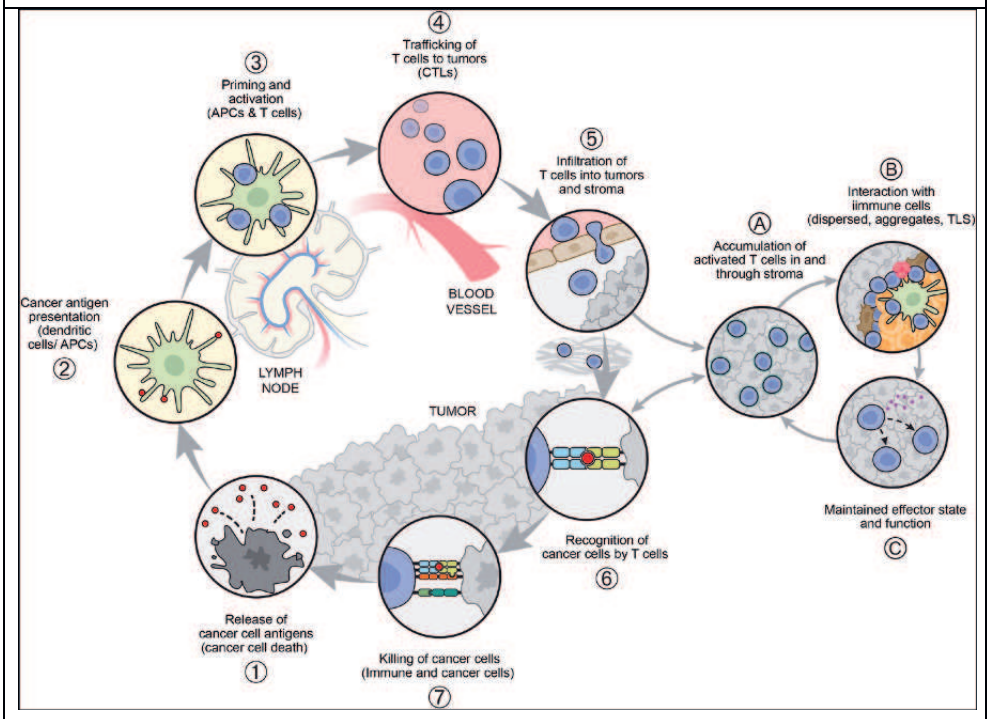


Dutch summary (Nederlandse samenvatting)

De belangrijkste focus van het onderzoek wat in dit proefschrift wordt beschreven ligt op immuun modulatie bij kankertherapie. Er is veel veranderd in het landschap van kankerbehandeling sinds de National Cancer Act van 1971, toen de Amerikaanse president Richard Nixon 'de oorlog tegen kanker' verklaarde [1]. Kankertherapiemodaliteiten omvatten onder meer chirurgie, bestralingstherapie, chemotherapie, hormonale therapie, gerichte therapie, immunotherapie en antilichaam-geneesmiddelconjugaten (ADC). Ondanks de vooruitgang in deze therapeutische modaliteiten blijft er een aanzienlijke behoefte aan verder onderzoek en verbetering, aangezien een aanzienlijk aantal patiënten na een diagnose van kanker aan hun ziekte blijft bezwijken. In het immunotherapeutische tijdperk van de behandeling van kankerpatiënten zijn verschillende manieren bestudeerd om de functionaliteit van het immuunsysteem te verbeteren. De kanker-immuniteitscyclus (CIC) biedt een raamwerk om de reeks gebeurtenissen te begrijpen die immuunreacties tegen kanker genereren (Figuur 1) [2].

Figuur 1. De kanker-immuniteitscyclus [2]



De CIC kan worden onderverdeeld in opeenvolgende kritische stappen, te beginnen met; Stap 1: het vrijkomen van kankerantigenen;
Stap 2: presentatie van kankerantigeen;
Stap 3: priming en activering in secundaire lymfoïde organen;
Stap 4: transport van T-cellen naar tumoren;
Stap 5: infiltratie van T-cellen in tumoren;
Stap 6: herkenning van kankercellen door T-cellen;
Stap 7: het doden van kankercellen [3].

De CIC kan bij elke stap worden aangetast en immuun modulatie is gericht op het overwinnen van blokkades en het reactiveren van de CIC om de herkenning en uitroeiing van tumoren te vergemakkelijken. In dit proefschrift hebben we verschillende therapeutische strategieën bij borstkanker (BC) en gemetastaseerde nierkanker (mRCC) beschreven en hier zullen we hun effect bespreken binnen de context van de CIC. Chemotherapie, bestralingstherapie, ADC, oncolytische virussen en gerichte therapie kunnen apoptose van tumorcellen induceren, wat leidt tot een verhoogde afgifte van kankerantigeen en daarmee de eerste stap in de CIC vormt om het immuunsysteem te activeren. Het ADC Trastuzumab-Deruxtecan (T-Dxd) bindt zich specifiek aan Her2+ (kanker)cellen, wat leidt tot een gericht effect van de cytostatische deruxtecan component, resulterend in de selectieve dood van Her2+ tumorcellen en de daaropvolgende afgifte van kankerantigenen, samen met schade-geassocieerde moleculaire patronen (DAMP's) die DC kunnen activeren en zo een antitumorale T-celreactie kunnen veroorzaken (dat wil zeggen immunogene celdood). Uit de systematische review en meta-analyse in **Hoofdstuk 5** blijkt dat T-Dxd de meest effectieve behandelingsmodaliteit is, leidend tot de hoogste objectieve responspercentages (ORR) bij patiënten met Her2+ gemetastaseerde BC (mBC) en hersenmetastasen (BM), alhoewel de meta-analyse met voorzichtigheid moet worden geïnterpreteerd vanwege de heterogeniteit van de geïncludeerde onderzoeken en een daarmee samenhangend ernstig risico op vertekening (bias).
BM vormen een uitdaging voor de ontwikkeling van effectieve antikankertherapieën, omdat bekend is dat conventionele chemotherapie in de hersenen minder effectief is vanwege de bloed-hersenbarrière (BBB). Bovendien beschikken BM over mechanismen om immuun detectie te voorkomen: ze veranderen de expressie van T-celliganden en co-stimulerende moleculen, activeren en onderdrukken microglia, activeren immunosuppressieve tumor-geassocieerde macrofagen (TAM), scheiden ontstekingsremmende cytokines af, reguleren eiwitten die nodig zijn voor de presentatie van antigeen, en verhogen de expressie van angiogene factoren [4]. Niettemin kan de BBB, vanwege de lekkende aard ervan bij kanker, worden gekruist door T-cellen en antilichamen, wat blijkt uit tumorreacties die worden waargenomen na blokkade van immuuncheckpoints.

T-Dxd wordt momenteel uitgebreider bestudeerd bij patiënten met Her2+ mBC en BM, en we wachten op de resultaten van de DESTINY Breast12-studie, inclusief patiënten met stabiele BM (ClinicalTrials.gov-identifier: NCT04739761) [5] en de HER2CLIMB-4-studie, waarbij de combinatie van tucatinib en T-Dxd werd onderzocht bij patiënten met en zonder BM (ClinicalTrials.gov-identifier: NCT04539938) [6]. Sinds de publicatie van onze meta-analyse zijn de bijgewerkte resultaten van de Tuxedo 1-studie gepubliceerd, waarin patiënten met Her2+ mBC en actieve BM werden onderzocht. Deze studie toonde een ORR aan van 83,3% bij de eerste 10 patiënten (inbegrepen in de meta-analyses), de bijgewerkte analyse van 15 geïncludeerde patiënten liet een iets lagere ORR zien van 73,3% [7]. Dit zal de resultaten van de meta-analyse echter niet significant beïnvloeden en we blijven nog steeds van mening dat T-Dxd het meest effectieve medicijn is dat is onderzocht bij patiënten met Her2+ mBC en BM.
Zoals eerder vermeld zou een verklaring voor de immunologische effecten van de ADC T-Dxd de afgifte van kankerantigenen op immunogene wijze kunnen zijn. Een andere factor zou kunnen zijn dat BC-cellen die een hoge Her2-expressie vertonen, een neerwaartse regulatie van de MHC klasse I-expressie hebben, waardoor de herkenning van CD8+ T-cellen wordt geremd [8]. Blokkering van de Her2-receptor door T-Dxd zou de expressie van MHC klasse 1 kunnen herstellen en daardoor de herkenning van T-cellen kunnen bevorderen (Stap 6 CIC, herkenning van kankercellen door T-cellen). Ook is bekend dat de trastuzumab-verbinding van T-Dxd een effect heeft via antilichaamafhankelijke celgemedieerde cytotoxiciteit (ADCC) [9], wat een rol impliceert voor T-Dxd in stap 7 van de CIC (doden van kankercellen). Sommige ADC's, geconjugeerd met tubulysine of pyrrolobenzodiazepinedimeer, vertoonden immuun activerende effecten en winst in combinatie met checkpointremmers (CPI) in immuun competente BC-muismodellen [10]. In deze muismodellen werd aangetoond dat de toediening van T-Dxd leidde tot zowel een toename van tumor-infiltrerende geactiveerde dendritische cellen (DC) en CD8+ T-cellen als tot een verhoogde PD-L1- en MHC klasse I-expressie op tumorcellen [10]. T-Dxd werd in deze muismodellen gecombineerd met respectievelijk een anti-PD1-antilichaam of een CTLA-4-antilichaam en beide combinaties werden als effectiever beschouwd dan beide monotherapieën [10, 11].

Helaas liet de toevoeging van nivolumab aan T-Dxd in een open-label, multicenter, fase 1b-onderzoek geen waarneembaar voordeel zien in de latere lijn setting van 48 patiënten met mBC (Her2+ n=32; Her2 laag n=16) [12]. Naast deze bovenstaande hypothesen en muisstudies is er eigenlijk weinig bekend over de effecten van T-Dxd op de immuunrespons, aangezien de meeste klinische onderzoeken geen immuun monitoring programma's hebben om immunologische translationele gegevens te verkrijgen. Het zou zeer interessant zijn om de immunologische effecten van T-Dxd bij patiënten met mBC verder te bestuderen en strategieën te onderzoeken om het immuunsysteem te versterken, geïllustreerd door de veelbelovende effecten van ADC gecombineerd met CPI bij blaaskanker [13]. ADC's zijn een ideale optie voor combinatietherapie omdat ze zich selectief kunnen richten op kankercellen (die bijvoorbeeld Her2 tot expressie brengen) en normale cellen kunnen sparen. De aanzienlijke omvang en het hydrofiele karakter van geconjugeerde antilichamen verminderen de niet-specifieke absorptie aanzienlijk, waardoor de specificiteit en veiligheid van ADC's wordt vergroot [14].
Om een T-celreactie tegen kanker te bewerkstelligen, moeten DC antigenen via MHC klasse I en MHC klasse II aan T-cellen presenteren. Conventionele DC type 1 (cDC1) zijn de belangrijkste CD8+ T-cel initiators via MHC klasse I, terwijl cDC2 meer geneigd zijn CD4+ T-cellen te stimuleren via MHC klasse II [15]. DC lijken echter veel complexer te zijn dan puur antigeenpresentatoren en initiators van een T-celreactie. DC fungeert ook als de belangrijkste bron van PD-L1 en is daardoor verantwoordelijk voor het ondersteunen en in stand houden van T-celreacties op PD-(L)1-blokkade [2]. Tumoren die niet erg gevoelig zijn voor het immuunsysteem (immuun-uitgesloten) missen niet alleen T-cellen in de tumormicro-omgeving (TME), maar missen ook DC. De rol van DC in de TME is niet beperkt tot de overdracht van antigenen van de tumor naar de tumor drainerende lymfeklieren (TDLN), maar ook om de aantrekking, activering en expansie van antigeen specifieke T-cellen in de tumor zelf te garanderen (stap 3 van de CIC, priming en activatie in secundaire lymfoïde organen en ook substappen 5A-C, priming en activatie van de lokale TME) [2].

In de fase 3 Spinoza studie, beschreven in **Hoofdstuk 4**, probeerden we DC te stimuleren om een robuuster immuunrespons te creëren bij de behandeling van patiënten met lokaal gevorderde BC (LABC) door de toevoeging van groeifactor GM-CSF aan neoadjuvante chemotherapie (NAC). BC wordt over het algemeen beschouwd als een immuun-uitgesloten tumortype en zou daarom baat kunnen hebben bij activering van DC's, vooral omdat LABC een groep patiënten omvat met een slechte prognose met een hoog risico op micrometastasen vanwege hun grote tumoromvang, invasie van nabijgelegen weefsels en/of verspreiding naar regionale lymfeklieren. We toonden inderdaad een verbeterde systemische DC differentiatie aan bij patiënten met LABC na NAC, ondersteund door GM-CSF of G-CSF. Met name bleek GM-CSF krachtiger te zijn in het stimuleren van de rijping van DC in TDLN dan G-CSF. Door GM-CSF ondersteunde NAC zou dus mogelijk een robuustere anti-tumor immuunrespons kunnen creëren dan de huidige op G-CSF gebaseerde NAC-strategieën. Systemische concentraties van GM-CSF en de duur van de blootstelling aan GM-CSF zijn belangrijk om rekening mee te houden bij het ontwerpen van toekomstige studies, omdat deze factoren de door GM-CSF geïnduceerde immunologische effecten zouden kunnen beïnvloeden [16, 17].
De meest succesvolle en revolutionaire immunotherapiebenadering tot nu toe bestaat uit de (goedgekeurde) CPI die zich richten op geprogrammeerde celdood 1 (PD-1), geprogrammeerde celdoodsligand (PD-L1), cytotoxische T-lymfocyt-geassocieerde antigeen 4 (CTLA-4), en meer recentelijk LAG-3 [18]. Deze CPI blokkeren controlepunteiwitten, zoals PD-L1 op tumorcellen en PD-1 op T-cellen, waardoor de T-cellen tumorcellen kunnen doden. CPI hebben hun effect in stap 3 (priming en activering in secundaire lymfoïde organen) en stap 7 (doden van kankercellen) van de CIC. In dit proefschrift werd mRCC onderzocht in een tijd dat CPI nog niet goedgekeurd waren voor deze indicatie. Sunitinib, een tyrosinekinaseremmer (TKI), was de standaard eerstelijnsbehandelingsoptie [19], gevolgd door everolimus, een mTOR-remmer, als een standaard tweedelijnsbehandelingsoptie [20].

Sinds 2018 zijn er verschillende opties toegevoegd aan het therapeutische arsenaal bij mRCC-patiënten, waaronder (a) de combinatie van de anti-PD1 CPI nivolumab plus de anti-CTLA-4 CPI ipilimumab [21], (b) de anti-PD-1 CPI pembrolizumab plus axitinib [22], (c) nivolumab plus cabozantinib [23] en (d) pembrolizumab plus lenvatinib [24].
Het kiezen van de beste therapeutische strategie voor mRCC is door al deze opties moeilijker geworden, waarbij keuzes worden afgewogen tussen effect, responstijd, toxiciteit en kosten. Inzicht in de redenen voor de verschillende behandelingsmodaliteiten kan helpen bij het nemen van beslissingen. Helaas ontbreken bij de meeste grote klinische onderzoeken uitgebreide immuun monitoring gegevens. Veel subsets van immunologische cellen brengen CTLA-4 en/of PD-1 en/of PD-L1 tot expressie en immuun monitoring programma's zouden kunnen helpen bij de detectie van biomarkers of een biomarkerprofiel kunnen creëren voor patiënten met een verwachte goede respons op CPI.
In het verleden, toen er minder effectieve behandelingsopties voor mRCC waren, probeerden we de toenmalige standaard tweedelijnsbehandeling met everolimus te verbeteren. Er werd aangetoond dat everolimus meerdere subgroepen van immuuncellen beïnvloedt, vooral Tregs, en dat het de balans doet doorslaan ten gunste van immuunsuppressie [25]. Tregs kunnen de activering, expansie en functie van andere T-cellen onderdrukken. Ze brengen CD4, CD25 en de aan immuunsuppressie gerelateerde transcriptiefactor Forkhead box P3 (Foxp3) tot expressie en zijn belangrijke bemiddelaars van immunosuppressieve reacties, waardoor auto-immuniteit wordt voorkomen. Een door Tregs gecreëerde immuunsuppressieve omgeving kan een ontoereikende antitumorreactie opleveren, waardoor de ideale situatie ontstaat waarin kankercellen zich kunnen ontwikkelen en groeien. Tumorcellen of TAM's produceren chemokinen (bijv. C-motief chemokine ligand 22 (CCL22)) om aan antitumorimmuniteit te ontsnappen door Tregs aan te trekken, b.v. via C-C chemokine receptor 4 (CCR4) [26, 27]. De hoeveelheid circulerende en (peri)tumorale Tregs worden geassocieerd met een slechtere overleving bij kankerpatiënten [28]. Een strategie bij mRCC om te sturen naar een meer immuun stimulerende combinatietherapie door de door everolimus geïnduceerde Treg-toename tegen te gaan, bij voorkeur door daadwerkelijke uitputting van Tregs, werd (en wordt) beschouwd als een aanpak die de moeite waard is om te onderzoeken. Uitputting van Tregs kan op verschillende manieren worden bereikt.

Uit preklinische studies is er bewijs dat het blokkeren van IL-2 of CD25, de op het celoppervlak tot expressie gebrachte α-keten van de IL-2-receptor, en het blokkeren van de CCR4-receptor of zijn ligand CCL22, effectieve strategieën zijn om de hoeveelheid en functionaliteit van Tregs te verminderen [26, 29-31].
Daarom omvatten klinische fase 1-2-studies gericht op het uitputten van Tregs het anti-CCR4 monoklonale antilichaam (mAb) Mogalizumab [32], (varianten van) het anti-CD25 mAb Daclizumab [33-35] en ONTAK, een IL2/difterietoxine conjugaat [36-41]. Andere manieren om Tregs uit te putten zouden kunnen worden bereikt door de toediening van het door glucocorticoïden geïnduceerde tumornecrosefactorreceptor-gerelateerde proteïne (GITR) antilichaam TRX518, waardoor GITR wordt geneutraliseerd, dat bij voorkeur tot expressie wordt gebracht op Tregs [42] of door de Foxp3-genexpressie te downreguleren door de histondeacetylase (HDAC)-remmer eninostat [43]. Een ander onderzoek was gericht op het in grote lijnen remmen van ontstekingen door Cox-2 te blokkeren, waardoor de CCL22-niveaus werden verlaagd en vervolgens de rekrutering van Treg werd belemmerd, door de toediening van NSAID M2000 [44]. Sinds de introductie van de anti-CTLA-4 CPI zijn er rapporten geweest die wijzen op hun rol bij het uitputten van Tregs, mogelijk via ADCC, hoewel de resultaten niet uniform zijn [45, 46]. Een andere benadering om Tregs uit te putten bestaat uit de toediening van metronomisch cyclofosfamide, een alkyleringsmiddel van het stikstofmosterdtype, dat Tregs (en niet T-helpercellen of cytotoxische T-cellen) selectief kan uitputten, hoogstwaarschijnlijk door een DNA-reparatiedefect te veroorzaken [47]. Er zijn verschillende fase 1-2 klinische onderzoeken uitgevoerd met cyclofosfamide in verschillende doses en schema's als modulator om Tregs uit te putten [48-55]. Omdat er controverse bestond over de optimale Treg-depletiedosis en het optimale schema van cyclofosfamide, hebben we een klinische fase 1-studie uitgevoerd waarin everolimus werd gecombineerd met verschillende schema's van metronomisch oraal cyclofosfamide bij patiënten met mRCC. De resultaten hiervan worden beschreven in **Hoofdstuk 2**. We toonden aan dat selectieve en significante Treg-depletie in het perifere bloed kon worden bereikt wanneer patiënten met mRCC werden behandeld met een standaard therapeutische dosis van 10 mg everolimus, gecombineerd met 50 mg cyclofosfamide, eenmaal daags.

Na vier weken combinatiebehandeling namen de absolute Treg-aantallen en Treg-percentages af. Deze combinatie van eenmaal daags oraal 50 mg cyclofosfamide en 10 mg everolimus werd geselecteerd voor het fase 2-deel van de studie. Er werden verschillende bijwerkingen (AE) geregistreerd, en de meest voorkomende bijwerkingen waren vermoeidheid, anorexia, huiduitslag, hoest, mucositis, misselijkheid, bloedarmoede en hypercholesterolemie. De totale incidentie van deze bijwerkingen lag in hetzelfde bereik als die van monotherapie met everolimus [20].
In **Hoofdstuk 3** wordt de fase 2 klinische studie beschreven waarin de combinatie van everolimus en metronomische cyclofosfamide werd geëvalueerd. Het onderzoek was gericht op het verbeteren van de progressievrije overleving (PFS) van patiënten met mRCC door de toevoeging van cyclofosfamide aan everolimus, vergeleken met everolimus monotherapie. De Treg-modulerende effecten van metronomisch cyclofosfamide waren vergelijkbaar met die waargenomen in het fase 1-deel van het onderzoek, helaas werd het fase 2-onderzoek bij de vooraf gedefinieerde tussentijdse analyse na inclusie van 24 patiënten stopgezet, omdat de PFS niet verbeterde van 50% naar de vooraf bepaalde grens van 70% bij 4 maanden. De immuun modulerende effecten van de combinatie van metronomisch cyclofosfamide en everolimus vertaalden zich niet in een veranderd klinisch resultaat, mogelijk als gevolg van het feit dat de Tregs die in het perifere bloed aanwezig waren, zich prolifereerden en sterke remmende functies hadden, wat een soort rekrutering van deze cellen impliceerde, wellicht als feedbackmechanisme. Dit zou gedeeltelijk kunnen verklaren waarom de waargenomen (tijdelijke) verlaging van Treg-niveaus niet voldoende was om effectieve en duurzame tumorimmuniteit te bereiken en daarom niet kon worden vertaald in een verbeterd klinisch resultaat.
Ondanks de beëindiging van het fase 2-gedeelte van de studie, hebben we relevante informatie verkregen uit de uitgebreide immuun monitoring gegevens waarmee rekening kan worden gehouden bij het ontwerpen van toekomstige immunotherapeutische benaderingen gebaseerd op mTOR-remmers. Bijvoorbeeld, mTOR-remming zou een interessante methode kunnen zijn bij het overwinnen van resistentie bij BRAF-gemuteerd melanoom door het remmen van de PI3K/AKT/mTOR-route, de ontwikkeling van Tregs zou dan een interessante prognostische sleutel kunnen zijn voor hernieuwde resistentie [56].

Bovendien kunnen de immuunmodulerende effecten van behandeling met cyclofosfamide, zoals beschreven in dit proefschrift, van extra waarde zijn, indien gecombineerd met CPI, gelijktijdig of opeenvolgend. We zagen een afname van het aantal immuun regulerende NK-cellen en een toename van het aantal cytotoxische natural killer (NK)-cellen door de toevoeging van cyclofosfamide. Bovendien induceerde cyclofosfamide een vermindering van het aantal van myeloïde afgeleide suppressorcellen (MDSC). Er is gesuggereerd dat het falen van CPI gedeeltelijk kan worden toegeschreven aan de aanhoudende onderdrukkende rol van MDSC of de variabiliteit in activering van NK-cellen [57, 58]; beide zouden kunnen worden tegengegaan door de toevoeging van cyclofosfamide.
Tegenwoordig is het gebruik van everolimus monotherapie voor mRCC grotendeels verlaten, vanwege de introductie van effectievere therapeutische alternatieven. Everolimus wordt nog steeds gebruikt in combinatie met lenvatinib, wat een verhoogde werkzaamheid laat zien in een fase 2-onderzoek [59]. Een mogelijk mechanisme dat de verworven resistentie tegen everolimus ondersteunt, zou kunnen zijn dat het remmen van mTORC1 door everolimus resulteert in mTORC2-afhankelijke activering van AKT met de STAT3- en ERK-routes en upregulatie van hypoxie-induceerbare transcriptiefactoren (HIF) [60]. Wanneer lenvatinib werd gecombineerd met everolimus, kon lenvatinib een van deze resistentiemechanismen van everolimus gedeeltelijk tegengaan door VEGF te blokkeren en daardoor HIF te downreguleren.

Slotopmerkingen

De verschillende therapeutische strategieën die in dit proefschrift voor BC en mRCC worden beschreven, hebben hun voorspelde impact in een of meer stappen van de CIC. Het begrijpen van de mogelijke effecten van nieuwe kankerstrategieën op immunologisch niveau is van cruciaal belang om vooruitgang te boeken in de richting van effectievere therapieën voor alle kankerpatiënten. Vooral tumoren die immuun-uitgesloten fenotypen vertonen vormen een grote uitdaging bij de ontwikkeling van deze immunotherapeutische strategieën. Wij zijn van mening dat zowel toekomstige vroege fase als gerandomiseerde fase 3 onderzoeken naar kankertherapie, translationele analyses zouden moeten omvatten, omdat nieuwe immunotherapeutische concepten effectiever zouden kunnen zijn op basis van de kennis van verschillende immunotypes en het CIC-raamwerk. Translationeel onderzoek zou ons kunnen helpen bij het begrijpen van de verschillende immunologische effecten van behandelstrategieën en het vinden van de juiste (dosis-aangepaste) combinatietherapie om zo meer soorten kanker te effectief te kunnen behandelen bij patiënten met tumoren van diverse immunotypes.

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About the Author (Curriculum Vitae)

Ingrid Michelle Werter (roepnaam Inge) werd geboren op 14 mei 1986 in Eindhoven. Nadat zij in 2004 haar tweetalig Gymnasium aan BC Broekhin Roermond afgerond had, startte zij met de studie geneeskunde bij universiteit Maastricht. Toen zij in 2010 haar geneeskundestudie voltooide ging zij een jaar in het Zaans Medisch Centrum te Zaandam als arts-assistent niet in opleiding (ANIOS) interne geneeskunde werken. Vervolgens startte zij haar opleiding tot internist en werkte zij vanaf 2012 gedurende 2 jaar in het Amstelland ziekenhuis te Amstelveen onder supervisie van dr. Bert Voerman. In 2014 continueerde zij haar opleiding tot internist in het VU medisch centrum in Amsterdam onder supervisie van prof. dr. Yvo Smulders. Als onderdeel van haar opleiding tot internist werkte zij in 2015 gedurende 4 maanden als chef-de-clinique in het Diakonessenziekenhuis in Paramaribo, Suriname. In 2016 onderbrak zij haar opleiding tot internist gedurende een jaar voor de start van haar promotieonderzoek in het Cancer Center Amsterdam onder leiding van prof. dr. Tanja de Gruijl en prof. dr. Hans van der Vliet. Na dit jaar onderzoek continueerde zij haar opleiding tot internist, binnen het aandachtsgebied medische oncologie in het VU medisch centrum, Amsterdam onder supervisie van dr. Inge Konings. Gedurende het aandachtsgebied oncologie doorliep zij ook een basiskwalificatie-onderwijs (BKO) leerlijn en behaalde hiermee een academische onderwijs gradering. Haar promotieonderzoek bleef zij zowel tijdens als na haar opleiding combineren met klinisch werk. Na het afronden van haar opleiding tot internist-oncoloog in 2019 werkte zij gedurende een jaar in het VU medisch centrum, Amsterdam en vervolgens 13 maanden in het Amphia ziekenhuis in Breda. Sinds november 2021 werkt ze als internist-oncoloog in het Rijnstate ziekenhuis te Arnhem. Inge is getrouwd met Wouter Geerling en zij hebben samen drie kinderen Casper (2018), Sebastiaan (2021) en Tobias (2025). Zij wonen in Arnhem.

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