TITLE: A phase 1b clinical trial optimising regulatory T cell depletion in combination with platinum-based chemotherapy in thoracic cancers
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ABSTRACT

Background: Single-agent cyclophosphamide can deplete regulatory T-cells (Treg). We aimed to determine optimal dosing and scheduling of oral cyclophosphamide, alongside pemetrexed-based chemotherapy, to deplete Treg in mesothelioma or non-small-cell lung cancer patients.

Methods: 31 Patients received pemetrexed +/- cisplatin or carboplatin on day 1 of a 21-day cycle (maximum 6 cycles). From cycle two, patients received cyclophosphamide, 50 mg/day, with intrapatient escalation to maximum 100/150 mg/day alternately. Immunological changes were examined by flow cytometry. Primary endpoint was Treg proportion of CD4+ T-cells, with doses tailored to target Treg nadir <4%.

Results: Reduction in Treg proportion was observed on day 8 of all cycles, and was not augmented by cyclophosphamide. Few patients achieved the <4% Treg target. Treg proliferation reached nadir one week after chemotherapy, and peaked on day 1 of the subsequent cycle. Efficacy parameters were similar to chemotherapy alone. 17% of patients ceased cyclophosphamide due to toxicity.

Conclusions: Specific Treg depletion to the degree seen with single-agent cyclophosphamide was not observed during pemetrexed-based chemotherapy. This study highlights the poor evidence basis for use of cyclophosphamide as an immunotherapeutic in combination with chemotherapy, and the importance of detailed flow cytometry studies.

Trial registration:

Clinical trial registration: www.anzctr.org.au identifier is 12609000260224.
KEYWORDS: malignant mesothelioma, non-small cell lung cancer, clinical trial, phase I, cyclophosphamide, immunotherapy, chemoimmunotherapy
1.0 INTRODUCTION

The alkylating agent cyclophosphamide has been used for decades as both a conventional cytotoxic agent and as an immunostimulatory agent in experimental treatment of cancer, predominantly in combination with immunotherapy [1,2]. The immunopotentiating effects of cyclophosphamide were originally identified as due to removal of a suppressive T cell population, now characterised as regulatory T cells (Treg) [3,4]. Over the past decade, there has been an increasing appreciation of the immunostimulatory effects of many other cytotoxic chemotherapies [5-8]. Mechanisms of immunopotentiation include enhancing the immunogenicity of cell death, the phenomenon of homeostatic proliferation with skewing of the regenerated lymphoid compartment towards anti-tumour response, and modulation of immune regulators including Tregs, myeloid derived suppressor cells (MDSC), and dendritic cells (DCs) – reviewed in [9].

Several studies have reported a preclinical phenomenon whereby low-dose cyclophosphamide enhances immune responses, including against tumours, by inhibition or depletion of Tregs [10,11]. In a murine model of mesothelioma, we found that the efficacy of cytotoxic chemotherapy could be enhanced by Treg depletion, and that cyclophosphamide preferentially depleted the cycling (Ki-67hi) CD4+ T cells [12]. (Ki-67hi) CD4+ T cells expressed increased levels of markers associated with a suppressive phenotype, Tumour Necrosis Factor Receptor 2 (TNFR2) and Inducible T cell COStimulator (ICOS). These data suggest that combining cytotoxic chemotherapy with cyclophosphamide to deplete Treg may potentiate the efficacy of chemotherapy.
In an independent study, Ghiringhelli et al administered low-dose oral cyclophosphamide to patients with advanced cancer using a metronomic schedule [13]. They demonstrated a profound reduction in numbers and proportion of CD3⁺CD4⁺CD25^{high} Tregs after 30 days treatment (100 mg daily), in both patients with advanced cancer and healthy volunteers, which was not observed in the remainder of the lymphocyte population. The proportion of Treg as a percentage of CD4⁺ T cells reduced to below 4% in a majority of patients treated. Furthermore, T cell and NK cell function was preserved or enhanced. Higher doses (200 mg/day) produced a broader lymphocyte depletion, highlighting a narrow optimum dose window [13].

The aim of this clinical trial was to determine the optimal safe and tolerable dose and schedule of oral metronomic cyclophosphamide to achieve maximal Treg depletion in people receiving cytotoxic chemotherapy for advanced thoracic cancers. Additional aims were to characterise the immune milieu during such treatment. A secondary aim was to identify any signal for enhanced efficacy of chemotherapy in combination with low-dose iterative oral cyclophosphamide. Patients with both mesothelioma and advanced non-small cell lung cancer (NSCLC) were included, due to the use of pemetrexed-based regimens in both settings.

2.0 PATIENTS AND METHODS

2.1 Study design

This was a prospective, single-centre, phase Ib trial of pemetrexed-based chemotherapy combined with iterative oral low-dose cyclophosphamide. The study was designed to allow intra-patient dose escalation of cyclophosphamide based on real-time monitoring of Treg proportions, aiming to identify the dose at which the nadir
Treg proportion was reached in individual participants. The primary endpoint was depletion of CD4^+CD25^+CD127^{lo}Foxp3^+ Tregs, expressed as the proportion of CD4^+CD25^+CD127^{lo}Foxp3^+ T cells in the total CD4^+ T cell pool after treatment. Secondary endpoints included depletion of cycling (Ki67^{hi}) Tregs, toxicity, objective tumour response as measured by the modified RECIST criteria for mesothelioma (mRECIST) [14] or by the RECIST criteria for NSCLC [15], time to progression (TTP), overall survival (OS), and treatment given. Treatment toxicities were graded using the National Cancer Institute (NCI) Common Toxicity Criteria AE, version 3.0. Extensive correlative immunological testing was performed with weekly blood draws for cryopreservation and subsequent analysis.

2.2 Ethical approval and ethical standards

This trial was prospectively registered with the Australia New Zealand Clinical Trials Registry (ACTRN12609000260224). The Sir Charles Gairdner Hospital institutional Human Research Ethics Committee reviewed and approved the protocol and all protocol amendments, informed consent documents and written study materials before their use. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants provided written informed consent prior to study enrolment or study procedures and agreed on the use of data for research and publication, which is fully anonymised.

2.3 Eligibility Criteria
Eligible patients had a histologically or cytologically confirmed diagnosis of malignant pleural mesothelioma (MPM) or locally advanced or metastatic non-small cell lung cancer (NSCLC), Eastern Co-operative Oncology Group (ECOG) performance status (PS) 0-1, and were planned for palliative-intent treatment with pemetrexed-based chemotherapy. Treatment could be first, second or third-line. All participants had measureable or evaluable disease on thoracic computed tomography (CT) scan. All had adequate haematological parameters, renal function, and hepatic function. Patients were ineligible if they were having concomitant treatment with other investigational agents or immunotherapy, had radiotherapy to all measurable lesions, a history of severe autoimmune disease, or a previous or concurrent malignancy within the past 5 years. Patients requiring oral corticosteroids for more than 5 days per chemotherapy cycle (ie. more than platinum agent supportive care medication) were ineligible. Pregnant or lactating women and patients with other serious medical disorders were also ineligible. The protocol was approved by the Institutional Human Research Ethics Committee and all patients provided written informed consent. Funding to conduct the clinical trial was provided by the National Health and Medical Research Council Australia. Clinical trial registration number (Australia New Zealand Clinical Trials Registry) ACTRN12609000260224.

2.4 Treatment Administration
The following pemetrexed-based chemotherapy regimens were allowed: Pemetrexed 500 mg/m² on day 1 of a 21-day cycle; cisplatin 75 mg/m² and pemetrexed 500 mg/m² day 1 of each 21-day cycle; or carboplatin AUC 4-6 and pemetrexed 500 mg/m² day 1 of each 21-day cycle. Dose reductions were not permitted on the first cycle, and patients requiring up-front dose reduction were ineligible. Subsequent dose reductions
were based on clinical and haematological parameters according to usual practice. Patients received vitamin B12 and folate supplementation as per usual practice with pemetrexed, and chemotherapy was continued to a maximum of six cycles. Standard prophylactic medications including corticosteroids (days -1 to 2-4 of each cycle) and antiemetics were given; however other concomitant cytotoxic, radiotherapy, surgery or investigational anti-cancer treatments were not allowed. Pemetrexed-based chemotherapy was stopped before 6 cycles in the event of progression, unacceptable toxicity, or patient withdrawal. In the event of chemotherapy cessation before 6 treatment cycles, cyclophosphamide was also stopped.

All patients received the first chemotherapy cycle without oral cyclophosphamide, in order to monitor the effects of chemotherapy alone on immunological parameters. Cyclophosphamide treatment was given to two separate patient cohorts (Fig 1A). Cohort 1 received oral cyclophosphamide days 1-14 of the 21-day cycle for each cycle after cycle 1. This seven day treatment-free period was initially included to ameliorate concerns over the potential for additional myelosuppression when cyclophosphamide was combined with chemotherapy. Once safety was established in Cohort 1, Cohort 2 received oral cyclophosphamide continuously throughout each cycle except cycle 1. The treatment doses for cohort 1 were: 50 mg/day; alternating 50/100 mg/day; 100 mg/day, and alternating 100/150 mg/day. The treatment doses for cohort 2 were: 50 mg/day; alternating 50/100 mg/day; 100 mg/day. Each dose level was continued for at least 1 cycle. Dose levels were escalated in individual patients (intrapatient dose escalation) until CD4+CD25+CD127loFoxp3+ Tregs reached a nadir of ≤ 4% of all peripheral blood CD4+ T cells during that cycle, as measured in real-time, at which point the dose remained at that level. This level was selected as achievable on the
basis of the literature (in particular, the data from Ghiringhelli and colleagues) and our own preliminary data [13]. If Treg proportions did not decrease to \( \leq 4\% \) of all peripheral blood CD4\(^+\) T cells, cyclophosphamide dose escalation was continued and further treatment cycles were given at the dose during which the Treg nadir was reached, even if this subsequently required de-escalation to a previous level. Where chemotherapy dose modification was required for toxicity, cyclophosphamide was the first drug to be discontinued. Haematological toxicity to \( \geq \) grade 3 mandated a decrease in cyclophosphamide dose to the previous dose level. If this occurred at the lowest cyclophosphamide dose level, cyclophosphamide was permanently discontinued. Allowance was made to withhold cyclophosphamide for the remainder of the cycle if day 8 or 15 blood tests revealed neutrophil count \(<0.75 \times 10^9/l\) or platelet count \(<50 \times 10^9/l\). Non-haematological toxicity of grade II or above, if considered related to cyclophosphamide, prompted a decrease in cyclophosphamide dose to the previous level. Cyclophosphamide was ceased in the event of haematologic or other grade III/IV toxicity requiring permanent discontinuation, or if the patient requested discontinuation of cyclophosphamide only. No post-protocol treatment recommendations were made.

Patients were reviewed clinically every 21 days whilst on treatment. Complete blood count and hepatic and renal function tests as well as assessment of toxicity by a study nurse were performed weekly whilst on treatment.

### 2.5 Assessment of Response

Clinical history and examination were required before study entry and day one each cycle. CT scan of the thorax and abdomen was performed at baseline, then weeks 6,
12, 18, and every 12 weeks thereafter. Radiological responses were assessed as above [14,15].

2.6 Assessment of time-to-event endpoints

Patients who died of their malignancy were considered to have disease progression at death. TTP was measured from date of enrolment to the first date of disease progression or death. Response duration was measured from the date of partial response (PR) to date of progression or death. Survival was defined as the time from study enrolment until death.

2.7 Correlative biomarkers

2.7.1 Blood collection and Peripheral Blood Mononuclear Cell (PBMC) isolation

Whole blood was collected into BD K_{2}EDTA Vacutainers (BD Diagnostics, Australia) weekly during treatment (ie. days 1, 8, and 15). A baseline sample was also collected at enrolment, within 14 days of day 1, in addition to pre-treatment on day one cycle one. All day one blood samples were collected prior to treatment administration. PBMC were isolated by Ficoll-Paque™ density gradient centrifugation following the manufacturer's instructions and cryopreserved in liquid nitrogen until analysis.

2.7.2 Flow cytometry

Whole blood was analysed by flow cytometry on the day of collection to obtain volumetric cell counts (cells per mL) of CD3^{+}CD8^{+} and CD3^{+}CD4^{+} T cells. Whole blood (10 µL) was stained for 60 min at room temperature in the dark using anti-CD3 PE, anti-CD4 Alexa Fluor 488, and anti-CD8 PECy7 antibodies (Supplementary Table 1). Fixation and red blood cell lysis was performed using BD FACS lysis buffer (BD
Biosciences), and data collected by three-color analysis using a Millipore Guava and Guava ExpressPro Software.

Realtime analysis of Tregs was performed immediately following weekly blood collection. For real-time analysis of peripheral blood CD4\(^+\) Tregs, 50 µL of whole blood was stained with anti-CD4 Alexa Fluor-488, anti-CD25-PECy5 and anti-CD127-PECy7 antibodies (Supplementary Table 1) for 60 min at room temperature, protected from light exposure. Red blood cells were lysed by the addition of 200 µL BD FACS lysing solution (BD Biosciences) for 15 min at room temperature and washed twice prior to intracellular staining.

Serial analyses were performed on cryopreserved PBMCs. All timepoint analyses were performed concurrently for individual patients to ensure comparability of experimental conditions across timepoints. PBMC were thawed in a 37°C water bath, and washed twice with PBS. For PBMC phenotyping 0.5-1 \(\times\) 10\(^6\) cells were stained. For identification of dead cells, samples were incubated with the Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) for 15 min at room temperature in PBS and protected from light. Cells were then washed and stained for expression of surface markers using antibodies against the following molecules: CD14-V500, CD19-V500, CD4-APC-H7, CD25-APC, CD127-PECy7. Surface staining was performed in the dark at room temperature for 25 minutes in Hanks Balanced Salt Solution without calcium, magnesium or Phenol Red (Life Technologies, Carlsbad, CA) supplemented with 2% heat inactivated FCS (Life Technologies) and 5 mM EDTA.

For intracellular staining, cells were fixed and permeabilized by incubation with 1X eBioscience Fixation/Permeabilization buffer (eBioscience) for 20 minutes at room
temperature in the dark. Samples were washed twice with 1X eBioscience Permeabilization buffer before staining with antibodies against FoxP3-PE and Ki67-FITC, in 1X eBioscience Permeabilization buffer for 45 min (whole blood) or 25 minutes (PBMC) at room temperature in the dark. Anti-Foxp3-PE staining was performed in the presence of 2% normal rat serum (eBioscience). Stained samples were stored in Stabilizing Fixative (BD Biosciences) prior to acquisition on a BD FACS Canto II equipped with 405 nm, 488 nm and 633 nm lasers (BD Biosciences) using Diva Software Version 8.0.1. The cytometer was routinely calibrated with BD cytometer setup and tracking (CS&T) beads (BD Biosciences). A minimum of 50,000 lymphocyte events were collected per sample. Single-stained and negative compensation beads (BD Biosciences) were used to calculate the compensation matrix. Cryopreserved PBMC from a single healthy donor were included with each staining run to control for inter-assay variability.

Flow cytometry data was analysed using FlowJo software (Treestar Inc, Ashland, OR). Positive staining and gating strategy were determined by comparison to fluorescence minus one (FMO) controls (Supplementary Fig 1).

2.8 Statistical considerations
Time to event endpoints were analysed using the method of Kaplan and Meier [16]. Summary statistics and frequencies were provided for each laboratory variable. Linear mixed models were used to analyse the relationship between time and lymphocyte subsets, in addition to testing for interaction. Data were analysed using the R environment for statistical computing and SPSS for Windows statistical package version 24.0.
3.0 RESULTS

3.1 Patient characteristics

Thirty-four patients were enrolled on the study over a 36-month period from June 2009 to June 2012; one diagnosis was subsequently revised to endometrial cancer, one patient was a screen failure, and one patient did not start treatment, leaving 31 eligible patients whose results are presented here (Fig 1B). Patient characteristics are shown in Table 1. All patients had radiologically assessable disease. Twenty-seven participants had MM, and four had NSCLC. All patients with NSCLC had stage IV disease. Staging of disease for participants with mesothelioma is shown in Table 1; no participants with mesothelioma had surgical treatment. None of the study participants received prior immunotherapy. The median time from diagnosis to study start was 3.1 months. The minimum follow-up was 28 months. Treatment was first-line for 27 patients; second-line for two patients, and third-line for two patients. Both second-line patients and one third-line patient had NSCLC.

3.2 Treatment Delivered

Forty-three cycles of cytotoxic chemotherapy alone and 101 cycles of chemotherapy in combination with cyclophosphamide were given. While all eligible patients (n=31) had cycle 1 treatment with chemotherapy alone, 29/31 had cycle 2 treatment with cyclophosphamide in combination with chemotherapy with two patients coming off-study before receiving any cyclophosphamide. Of these two patients, one withdrew from treatment through personal choice, and the other experienced disease progression via a new lesion. 12 cycles of chemotherapy without cyclophosphamide were given after cycle 1, with 5 patients ceasing cyclophosphamide but continuing
chemotherapy due to: haematological toxicities requiring cessation of cyclophosphamide as per protocol (n=3); grade 3 fatigue (n=1); and grade 2 nausea/patient wishes (n=1). Supplementary Table 2 shows the number of cycles given at each dose level whilst patients remained on-study.

Patients received a median of 4 cycles of chemotherapy (range 1-6). Dose reductions were required for both pemetrexed and cisplatin in one participant, and for both pemetrexed and carboplatin in one participant, with a median dose intensity of 100% for all of cisplatin, carboplatin, and pemetrexed.

3.3 Toxicity
Toxicities are shown in Table 2. Emesis was in keeping with reported toxicities of cisplatin/pemetrexed, as was fatigue; one patient experienced grade 3 fatigue. Stomatitis and dysgeusia were common. However, haematological toxicities were prominent, with 48% of patients experiencing grade 3 anaemia during study participation. Grade 3 and 4 neutropenia were reported in 32% of patients, although there were no episodes of febrile neutropenia whilst receiving cyclophosphamide.
Six serious adverse events were reported during the study, in five patients. One (febrile neutropenia) attributed to treatment during cycle one, three due to tumour, and two episodes of non-neutropenic fever requiring hospital admission which were not attributed to study drug. There were no permanent sequelae.

3.4 Response to Treatment
All eligible patients had measurable disease, but one withdrew from the study before the first evaluation. Eleven patients (37%) had a partial response and 15 (50%) had
stable disease as their best radiological response, with 4 (13%) progressive disease at first assessment (Fig 2A). All participants with follow-up data are deceased, with all deaths attributed to thoracic malignancy; one patient was lost to follow-up. Median OS was 17.4 months (95% CI 14.1 – 23.1 months), and median PFS was 6.6 months (95% CI 5.8-9.4 months) (Fig 2B). Upon subdividing subjects into either ‘epithelioid MPM’ (n=20) or ‘non-epithelioid MPM plus NSCLC’ (n=11) groups, the epithelioid MPM patients showed significantly better OS (20.1 mo vs 8.7 mo, p=0.0085), but not PFS (7.7 mo vs 6.3 mo, p=0.097). Treatment schedules, cyclophosphamide dosing, disease pathology and TTP for individual patients are shown in Fig 3. No association between either PFS or toxicities and cyclophosphamide treatment schedules was apparent.

3.5 Immunological biomarkers

All lymphocyte subset parameters showed a marked cyclical pattern, in keeping with anticipated changes in haematological parameters induced by chemotherapy alone. Realtime Treg (CD25^+CD127^{lo}Foxp3^{+}) proportion of total CD3^+CD4^+ T cells was assessed by flow cytometry, see supplementary Figure S1 for gating strategy. Contrary to our initial hypothesis, there was no durable reduction in the Treg% in peripheral blood over the course of cyclophosphamide treatment. A small-scale reduction (between day 1 and day 8) was observed directly after chemotherapy, followed by a rebound to baseline levels (between day 15 and day 1 of the following cycle) - repeated for each treatment cycle (Fig 4A). No significant difference in Treg% was observed between chemotherapy alone (cycle 1) versus chemotherapy plus cyclophosphamide (cycles 2-6). In keeping with this observation, the scale of absolute Treg depletion in terms of cell concentration did not decrease further than was
observed during the first cycle of treatment, even after cyclophosphamide was added to the treatment regime (Fig 4B). No significant differences in either proportion or concentration of Tregs were seen when comparing intermittent vs continuous cyclophosphamide scheduling. We observed a distinct cyclical pattern of Treg proliferation (Ki67+), with maximal proportions of proliferating Tregs consistently highest at day 1 of each treatment cycle (prior to receiving chemotherapy) and lowest at day 8 (one week after chemotherapy) (Fig 4C). No differences in Treg proliferation were observed between intermittent or continuous cyclophosphamide treatments. Multiple cycles of combination chemotherapy and cyclophosphamide did not deepen Treg depletion, with half (14/31) of patients showing a nadir in Treg% during the first cycle of triplet therapy (i.e. cycle 2) and around a quarter of patients (7/31) achieving Treg nadir during cycle one, prior to receiving cyclophosphamide (Fig 4D).

Before starting treatment, half of patients had serum mesothelin concentrations above the upper limit of normal defined as 2.5 nM (data not shown) [17]. There was no correlation between either baseline mesothelin levels, or the change in mesothelin levels over time with response to therapy or OS.

4.0 DISCUSSION
Cyclophosphamide is widely used in cancer treatment, both as a cytotoxic agent and in combination with immunotherapies. Results of clinical studies have varied widely; it is apparent that the immune effects of cyclophosphamide are dose-dependent, and differ in the context of other therapies [18,19].

The primary endpoint of this study was to identify the cyclophosphamide dose that optimised Treg depletion when used in the context of pemetrexed-based
chemotherapy. The study design is novel in that intra-patient dose escalation and de-
escalation was performed in real-time in response to the observed nadir Treg proportion. In cohort 1, while toxicity did not preclude cyclophosphamide doses escalating to the pre-specified maximum of alternating daily 100/150 mg, real-time analysis demonstrated a ’rebound’ in Treg proliferation when cyclophosphamide was ceased for one week each cycle. Furthermore, increasing cyclophosphamide doses did not result in additional patients reaching the goal nadir of ≤4% of total peripheral CD4\(^+\) cells. For cohort 2, a protocol amendment incorporated continuous cyclophosphamide, with the rationale that continuous suppression of Treg may avoid rebound Treg proliferation and increase the proportion of patients reaching the target nadir. The amendment also removed the final dose level (100/150 mg) as additional depletion was not seen at this level.

Since modulation of Tregs by cyclophosphamide during concomitant chemotherapy was our mechanistic goal for this treatment, we included an internal control for each patient using a first, chemotherapy-only, treatment cycle with which to individually benchmark subsequent triplet therapy. Our study did not show depletion of Tregs in response to addition of cyclophosphamide over that seen with pemetrexed-based chemotherapy alone. Indeed, the largest variation in Treg numbers occurred during cycle one. Cyclophosphamide did not induce Treg depletion which was more profound or durable that chemotherapy alone, and this was independent of whether patients received intermittent or continuous daily cyclophosphamide.

Previous work by our group in a mouse model of mesothelioma demonstrated a benefit of combining cyclophosphamide with gemcitabine chemotherapy. In this model,
subcutaneous tumours from the AB1-HA mesothelioma cell line demonstrated an immunosuppressive microenvironment, and Treg depletion was therapeutic [20]. Cyclophosphamide improved the efficacy of gemcitabine and selectively depleted T cells with a Ki67\textsuperscript{hi} population that expressed ICOS and TNFR2 – markers linked to a maximally suppressive phenotype – in tumour and tumour-draining lymph nodes [12,21-23]. However, neither selective depletion of general Tregs nor a Ki67\textsuperscript{hi} subpopulation were observed in this current clinical study, albeit our observations were in PBMC rather than tumour or lymph nodes due to clinical constraints. One notable variation between the clinical and preclinical studies is the choice of chemotherapy – with gemcitabine used in the mouse model and cisplatin/pemetrexed in the clinic, as this was the standard of care during the study. It is possible that the kinetics of cell cycle arrest are different between pemetrexed and gemcitabine in terms of depth and duration. Tregs generally rebound more strongly than other lymphocyte subgroups following general lymphodepletion [24,25]. Indeed, our results confirm an extreme reduction of proliferating Tregs one week after cisplatin/pemetrexed chemotherapy followed by a rebound back to or even exceeding pre-treatment levels. Thus, cisplatin/pemetrexed chemotherapy may not the ideal partner for low-dose cyclophosphamide. A number of other preclinical studies combining cyclophosphamide with immunogenic chemotherapy have reported positive results [26-28], but to date none have led to positive randomised clinical trials comparing treatment with cyclophosphamide vs no cyclophosphamide. In this regard, factors such as routes of administration, dosing regimens and differences in serum half-life of cyclophosphamide (<17 min vs 6.5 hr in mice and humans respectively) may all impact on the effectiveness of translation from mouse to man [18].
Several clinical trials have utilised cyclophosphamide as an immune modulator, either as monotherapy or in combinations. In patients with metastatic breast cancer, low-dose single-agent cyclophosphamide (50 mg/day) depleted Tregs only transiently and did not decrease their suppressive capacity, but was reported to induce stable tumour-specific T-cell responses [29]. A recent study in colorectal cancer observed delayed progression, along with increased numbers of tumour antigen-specific T cells producing both granzyme B and longer-term IFN-γ, following treatment with single-agent low-dose cyclophosphamide [30]. Metronomic cyclophosphamide may be a beneficial partner for vaccination strategies; a 2011 study found cyclophosphamide increased the effectiveness of an oncolytic adenovirus vaccine in comparison to vaccine alone in patients with metastatic solid cancers [31]. A more recent clinical trial in patients with mesothelioma combined low-dose cyclophosphamide with DC vaccines; ten patients received immunotherapy 10-17 weeks following either debulking surgery plus chemotherapy, or chemotherapy alone. In this context, cyclophosphamide transiently reduced not only the Treg proportion, but also the naïve and central memory CD4 T cell proportions within the overall CD4 T cell compartment. Proportions of effector memory CD4 T cells, and CD8 T cells relative to CD4 T cells, increased. Perhaps surprisingly, the proliferating (Ki67+) proportion of Tregs increased following cyclophosphamide treatment. However, as in our study, this was an early-phase trial with no control arm comprising patients who did not receive cyclophosphamide [32]. In a slightly different approach, low-dose cyclophosphamide has also been used in the clinic to counteract a treatment-induced Treg increase that occurs with some anti-cancer drugs, such as lenalidomide or everolimus, where an increase in Tregs has been considered possibly responsible for acquired treatment resistance [33,34]. There is preclinical evidence that cyclophosphamide can synergise
with immune checkpoint inhibiting antibodies, for example anti-PD1 [35,36]. Numerous investigations are currently in progress exploring the combination of metronomic cyclophosphamide either with immune checkpoint inhibitors or vaccination strategies (ClinicalTrials.gov), the results of which will inform future studies.

Whilst treatment toxicity was manageable and the addition of cyclophosphamide did not increase clinically significant myelosuppression, there was no strong clinical efficacy signal to support further use of this combination, with response rates being in keeping with previous reports (i.e. 45% in our study vs 41% for standard care platinum-based chemotherapy). Our PFS and OS are somewhat favourable, with an OS of 17.38 months compared to 18.8 months and 18.1 months in the MAPS (chemotherapy plus bevacizumab) and recently reported Checkmate 743 (nivolumab plus ipilimumab) studies, respectively [37,38]. However, this may be explained by patient selection and more restrictive inclusion criteria in this single centre phase Ib clinical trial and cannot be confidently attributed to the study treatment regimen. As a point of interest, and as may be expected, participants diagnosed with epithelioid MPM performed better than the group of non-epithelioid plus NSCLC study subjects in terms of OS but not PFS – however this may also be explained by the small number of data points once patients were subdivided into these two groups.

Although the vast majority (26/31) of patients in this study were 1st line mesothelioma, we do acknowledge that the heterogeneity of our cohort in terms of cancer type and line of treatment may be viewed as a limitation. Additionally, data on other subsets of immune cells, e.g. cytotoxic T cells, NK cells, DCs, and MDSCs, would be useful as it
may put our findings into a broader context of the overall changes occurring of our chemoimmunotherapy treatment.

5.0 CONCLUSION
The combination of pemetrexed-based chemotherapy with oral low-dose cyclophosphamide was tolerable and shows efficacy similar to that expected from chemotherapy alone. However, Treg-specific depletion to the degree seen with single-agent low-dose cyclophosphamide was not observed in the context of pemetrexed-based chemotherapy. Combinations of low-dose iterative cyclophosphamide are unlikely to enhance the efficacy of chemo-immunotherapy combinations through Treg depletion. This study highlights the current poor evidence basis for the use of cyclophosphamide with immunotherapy. There is a need to better study the use of both iterative low-dose and single high-dose cyclophosphamide in immunotherapy combinations using detailed flow cytometry and randomised clinical trials.
Legends for tables and figures:

Table 1. Patient demographics and baseline characteristics for 31 eligible patients.

Table 2. Adverse events occurring in all patients, all grades, all courses.

Figure 1. (A) Treatment schedules showing cyclophosphamide dosing from cycle 2 onward. Patients in cohort 1 had a one-week break from daily cyclophosphamide prior to the end of each treatment cycle, those in cohort 2 received continuous cyclophosphamide. Chemotherapy was given on day 1 of each treatment cycle for a maximum of 6 cycles. Bloods were taken on days 1, 8 and 15 of each cycle. (B) Flow diagram detailing patient recruitment and analysis according to CONSORT guidelines.

*patient retrospectively diagnosed with endometrial cancer.

Figure 2: (A) Kaplan-Meier plots showing overall survival and progression-free survival of all 31 patients. (B) Waterfall plot showing change in tumour size for 30 patients.

Figure 3: Swimmer plot showing individual patient treatment including disease pathology, duration/dose/treatment cohort of cyclophosphamide, time to progression and occurrence of treatment intolerances.

Figure 4: Longitudinal flow cytometry data in Tregs across six cycles of chemoimmunotherapy, for (A) the Treg proportion of CD4+ lymphocytes; (B) absolute concentration of Tregs in peripheral blood; and (C) the proliferating (Ki67+) proportion of Tregs. Left-hand panels show observed values from individual patients from both intermittent and continuous treatment cohorts, together with their empirical means (lines). Values below the X-axes denote the treatment day within that
particular cycle (i.e. day 1, day 8, or day 15), plus number of cycles. Timepoint ‘B’
represent pre-study baseline samples. Right-hand panels show results of fitting a
linear mixed model; a linear trend over time and additive treatment effects of the day
of the treatment yield the corresponding population average curves. Average change
over the duration of the study is described. (D) Data from 31 patients showing timing
of Treg nadir during treatment.
References


*Evidence that cyclophosphamide may decrease not only the the number but also the suppressive function of Tregs.


*Characterisation of the dose-dependent bimodal effect of cyclophosphamide on the immune system.


**Clinical demonstration of the ability of single-agent metronomic low dose cyclophosphamide selectively depleting Tregs in a variety of solid tumour types.


Table 1: Patient demographics and baseline characteristics for 31 eligible patients

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<td>9.7</td>
</tr>
<tr>
<td>MM mixed/biphasic</td>
<td>3</td>
<td>9.7</td>
</tr>
<tr>
<td>MM unknown</td>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td>NSCLC Adenocarcinoma</td>
<td>4</td>
<td>12.9</td>
</tr>
<tr>
<td>Time since diagnosis (months)</td>
<td>2.4 (0.8-20)</td>
<td></td>
</tr>
<tr>
<td>Line of therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First (Meso; NSCLC)</td>
<td>27 (26;1)</td>
<td>87.0</td>
</tr>
<tr>
<td>Second (Meso; NSCLC)</td>
<td>2  (0; 2)</td>
<td>6.5</td>
</tr>
<tr>
<td>Third (Meso; NSCLC)</td>
<td>2  (1; 1)</td>
<td>6.5</td>
</tr>
<tr>
<td>Disease stage at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>III</td>
<td>9</td>
<td>29</td>
</tr>
<tr>
<td>IV (Meso; NSCLC)</td>
<td>13 (9; 4)</td>
<td>42</td>
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</table>
Table 2. Adverse events occurring in all patients, all grades, all courses

<table>
<thead>
<tr>
<th>TOXICITY</th>
<th>GRADE</th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Nausea</td>
<td>16</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Vomiting</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fatigue</td>
<td>18</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Anorexia</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>constipation</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stomatitis</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Watery eyes</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dizziness/lightheadedness</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Flu like symptoms</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Limb oedema</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Dysgeusia</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rash</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hearing loss</td>
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<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
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**Investigations**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Hemoglobin</td>
<td>8</td>
<td>9</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Platelet count</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>6</td>
<td>12</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>
FIGURE 1

A
Cohort 1: Intermittent CTX (n=11)
Chemotherapy

- Day 1
- Day 8
- Day 15
- Day 1

Blood samples taken

Cyclophosphamide

x≤6

Cohort 2: Continuous CTX (n=20)
Chemotherapy

- Day 1
- Day 8
- Day 16
- Day 1

Blood samples taken

B

34 patients consented and evaluated

- 2 deemed ineligible (12, 19)

32 had baseline samples collected

- 1 excluded from analysis (22)

31 included in final analysis
FIGURE 2

A

% change from baseline

- Progressive disease
- Stable disease
- Partial response

B

Overall Survival

Percent survival

Median 17.38

Progression Free Survival

Percent progression-free

Median 6.6
FIGURE 3
FIGURE 4

A

B

C

D

Average change in Treg over 20 time points, p=0.104
Difference between INT and CONT schedule 0.57, p=0.335

Average change in Treg, p=0.001
Difference between INT and CONT schedule 0.85, p=0.008
**Supplementary Table S1.** List of monoclonal antibodies used for flow cytometric staining. Abbreviations: Cat = catalogue, AF = AlexaFluor, m = mouse, r = rat, h = hamster.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluor</th>
<th>Clone</th>
<th>Isotype</th>
<th>Manufacturer</th>
<th>Cat #</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>CD3</td>
<td>PE</td>
<td>SK7</td>
<td>m IgG1</td>
<td>BD Biosciences</td>
<td>347347</td>
<td>1/20</td>
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<tr>
<td>CD4</td>
<td>AF488</td>
<td>RPA-T4</td>
<td>m IgG1</td>
<td>BD Biosciences</td>
<td>557695</td>
<td>1/50</td>
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<tr>
<td>CD4</td>
<td>APC-H7</td>
<td>RPA-T4</td>
<td>m IgG1</td>
<td>BD Biosciences</td>
<td>560158</td>
<td>1/40</td>
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<td>CD8</td>
<td>PECy7</td>
<td>RPA-T8</td>
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<td>555368</td>
<td>1/50</td>
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<td>CD14</td>
<td>V500</td>
<td>M5E2</td>
<td>m IgG2a</td>
<td>BD Biosciences</td>
<td>561391</td>
<td>1/80</td>
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<td>CD19</td>
<td>V500</td>
<td>HI19</td>
<td>m IgG1</td>
<td>BD Biosciences</td>
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<td>CD25</td>
<td>APC</td>
<td>M-A251</td>
<td>m IgG1</td>
<td>BD Biosciences</td>
<td>555434</td>
<td>1/5</td>
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<td>CD45RA</td>
<td>V450</td>
<td>HI100</td>
<td>m IgG1</td>
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<tr>
<td>CD127</td>
<td>PECy7</td>
<td>eBioRDR5</td>
<td>m IgG1</td>
<td>eBioscience</td>
<td>25-1278</td>
<td>1/100</td>
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<tr>
<td>Foxp3</td>
<td>PE</td>
<td>PCH101</td>
<td>r IgG2a</td>
<td>eBioscience</td>
<td>12-4776</td>
<td>1/20</td>
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<tr>
<td>Ki67</td>
<td>FITC</td>
<td>B56</td>
<td>s IgG1</td>
<td>BD Biosciences</td>
<td>556026</td>
<td>1/10</td>
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</tbody>
</table>
**Supplementary Table S2:** Total number of treatment cycles administered at each dose level.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Cohort 1 (day 1-14)</th>
<th>Cohort 2 (Continuous)</th>
<th>All patients</th>
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<tbody>
<tr>
<td>No cyclophosphamide</td>
<td>11</td>
<td>20</td>
<td>31</td>
</tr>
<tr>
<td>50 mg/day</td>
<td>12</td>
<td>38</td>
<td>50</td>
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<tr>
<td>50/100 mg alternate days</td>
<td>12</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>100 mg/day</td>
<td>7</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>100/150 mg alternate days</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
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</table>
Supplementary Figure 1: Flow cytometry gating strategy. A) Realtime analysis of Tregs in whole blood: CD4+ T cells are identified from within the lymphocyte population, CD25+CD127lo cells are subsequently gated for Foxp3 to identify Tregs. B) Batched analysis of cryopreserved PBMC: doublet discrimination identifies single cells, whereupon CD14, CD19 and a viability marker are used to exclude monocytes, B cells and dead cells. CD25+CD127loFoxp3+ Tregs are then identified from within the CD4+CD3+ T cell population. Proliferating Tregs are identified by expression of Ki67.