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Title Page

A single centre phase II trial to assess the immunological activity of TroVax® plus
pemetrexed/cisplatin in patients with malignant pleural mesothelioma – the SKOPOS trial

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Running title

Cancer vaccine and chemotherapy combination in mesothelioma

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Conflict of Interest

RH is employed by Oxford BioMedica, the manufacturer of TroVax® who supplied the vaccine for this trial. We declare that no other authors have any conflicts of interest.

Abbreviations:

AE, adverse events; BLCL, B lymphoblastoid cell line; DI, dose intensity; ELISA, Enzyme linked immunosorbant assay; FBC, full blood count; MDSC, myeloid derived suppressor cells; MPM, malignant pleural mesothelioma; NLR, neutrophil:lymphocyte ratio; ORR, objective response rate; OS, overall survival; PBMC, peripheral blood mononuclear cells; PFS, progression free survival; PPA, per protocol analysis; PS, performance status; RECIST, Response Evaluation Criteria in Solid Tumors; Treg, Regulatory T cells; WHO, World Health Organization; WT-1, Wilms tumor antigen-1.

Abstract (248 words)

Vaccines in combination with chemotherapy have been shown to be safe in different tumor types. We investigated the immunological activity of the TroVax[®] vaccine in combination with pemetrexed-cisplatin chemotherapy in malignant pleural mesothelioma (MPM).

In this first line, open-label, single-arm, phase 2 study, patients with locally advanced or metastatic MPM were enrolled. Eligible patients received up to 9 intramuscular injections of TroVax[®], starting two weeks before chemotherapy and continuing at regular intervals during and after chemotherapy to 24 weeks. The primary endpoint was the induction of cellular or humoral anti-5T4 immune response (defined as a doubling of either response at any of six follow-up time points), with a target response rate of 64%.

Of 27 patients, enrolled between Feb 2013-Dec 2014, 23 (85%) received at least three doses of TroVax[®] and one cycle of chemotherapy and were included in the per-protocol analysis (PPA). 22/23 patients (95.6%) developed humoral or cellular immune response to 5T4. Thus, the study reached its primary endpoint. Disease control was observed in 87% of patients (partial response: 17.4%, stable disease: 69.6%). **The median progression-free survival was 6.8 months and median overall survival 10.9 months.** Treatment-related adverse events were comparable to those observed in patients with chemotherapy alone. Translational immunology studies revealed a circulating baseline immune signature that was significantly associated with long-term (>20 months in n=8/23, 34.8%) survival.

In this phase 2 trial, TroVax[®] with pemetrexed-cisplatin chemotherapy showed robust immune activity, acceptable safety and tolerability to warrant further investigation in a phase 3 setting.

Introduction

Malignant pleural mesothelioma (MPM) is an incurable and fatal malignancy of the pleural membranes. MPM has a poor prognosis and patients have a median survival of 9-18 months in clinical trials.^{1,2} To date, only two randomized phase III trials in MPM have shown benefit for one systemic treatment approach over another. In the first of these trials,¹ 448 patients were randomized to chemotherapy using pemetrexed plus cisplatin or cisplatin alone. Median overall survival (OS) was significantly longer in the pemetrexed-cisplatin arm (12.1 vs. 9.3 months, $p < 0.02$). As a result of this trial, pemetrexed-cisplatin was established as the chemotherapy standard of care for patients with MPM. For this reason, we chose pemetrexed-cisplatin as the chemotherapy regimen for this trial. Subsequently, the addition of bevacizumab to pemetrexed-cisplatin has been shown to further increase median survival by two to three months compared to chemotherapy alone.² Despite the benefits seen from chemotherapy with or without the addition of bevacizumab, it is clear that new therapeutic strategies are urgently needed for MPM.

There is significant recent interest in the potential role of immunotherapy in the management of patients with MPM, which has been shown to respond to various immunotherapeutic strategies in animal models and early phase clinical trials.³ Spontaneous regression, associated with improved immune parameters has also been reported.⁴ Indeed, prognostic significance of intratumoral immune cell subsets in MPM have revealed CD8+ T cells and CD20+ B cells as positive prognostic indicators^{5,6}, whilst CD163+ macrophages and regulatory T cells (Treg) are negative indicators.^{6,7} Conversely, very few studies have addressed the significance of peripheral immune parameters to clinical outcome, though proliferating CD8+ T cells, co-expressing Ki67⁸ and dysfunctional dendritic cells⁹ have been described. Furthermore, blood parameters such as high white blood cell count, neutrophil

to lymphocyte ratio (NLR), monocyte numbers and high monocyte to lymphocyte ratios have also shown negative prognostic value.¹⁰⁻¹² This indicates that patient screening and stratification may improve clinical benefit, particularly in immunotherapy trials.

Current studies are testing a range of immunotherapeutic approaches, such as treatment with a mesothelin-targeting chimeric antibody (amatuximab), type-I interferon delivered by an adenoviral vector, intrapleural viruses and antigen-specific vaccines, such as the Wilms tumor antigen-1 (WT-1) vaccine.¹³ The WT-1 vaccine has shown evidence of activity in a trial of 40 patients with MPM randomized to WT-1 vaccine or observation after multimodality treatment. Immune checkpoint inhibitors have also been tested in MPM with mixed early results and their further clinical trials - single or combination treatments – are currently ongoing.^{14, 15}

5T4 is a 72 kDa oncofoetal glycoprotein that is expressed in many solid tumors but shows minimal or no expression in normal tissues.¹⁶ We have shown that 5T4 is widely expressed in mesothelioma tissue and on mesothelioma cell lines.¹⁷ Unlike WT-1 and mesothelin, which display subtype-restricted expression, often excluding the more aggressive sarcomatoid variant, 5T4 expression has been shown in all MPM subtypes.¹⁷ 5T4-specific T cell responses were demonstrated by patients' peripheral blood mononuclear cells (PBMC) and pleural fluid cells (Al-Taei at al., unpublished), making it a valid antigen for targeted therapies, including immunotherapy, in MPM.

TroVax® (Oxford BioMedica, Oxford, UK) is a therapeutic cancer vaccine which consists of a highly attenuated vaccinia virus (modified vaccinia Ankara) containing the 5T4 glycoprotein

gene. TroVax[®] has been administered to more than 500 patients with renal, colorectal and prostate cancer.¹⁸ These clinical studies showed that TroVax[®] is well-tolerated and induced 5T4-specific antibody and/or cellular immune responses in the majority of patients. In addition, TroVax[®] was also well tolerated when used in combination with chemotherapy in patients with colorectal cancer.¹⁹

Pemetrexed-cisplatin chemotherapy is seen as a UK and international standard of care for patients with MPM. We aimed to combine the TroVax[®] vaccine with first line pemetrexed-cisplatin chemotherapy in MPM patients in a single-arm, single-center, phase II trial (SKOPOS), in order to determine 5T4-specific antibody and/or cellular immune responses, activity, safety and feasibility. Furthermore, we carried out retrospective immunohistochemistry, full blood count (FBC) and immunophenotypic analyses to identify potential immune prognostic indicators.

Results

Patient and treatment details

Between Feb 2013, and Dec 2014, 29 patients were enrolled at Velindre Cancer Centre in Cardiff, UK. 23/29 patients (79%) received at least three doses of TroVax® and one cycle of chemotherapy and were included in the PPA (Figure 1). Six participants were not included in the PPA as four did not receive the minimum 3 injections, one patient was later found not to have mesothelioma, and one was found to have a co-primary cancer. Median age of the PPA participants was 66 years (IQR 61-70), 20/23 (87%) were male, 20/23 (87%) had epithelioid, 3/23 (13%) had sarcomatoid MPM, and 12/23 (52%) had WHO PS of 0 (Table 1).

The median number of TroVax® injections administered to PPA patients was nine (IQR 7-9): 16/23 (70%) patients received all nine injections, 6/23 (26%) patients received between four and nine injections, and 1/23 (4%) patients received three injections. The reasons that seven patients missed injections are follows: two patients died, two withdrew due to adverse effects (AE), one withdrew due to disease progression, one withdrew to undergo surgery for MPM, and one patient missed the week 11 injection. Four cycles of pemetrexed-cisplatin chemotherapy treatment were successfully administered to 19/23 (83%) patients. Four patients did not complete four cycles of chemotherapy: two died and two withdrew due to AE. The median number of chemotherapy cycles was four (IQR 4-4). Median cisplatin DI was 90% (IQR 75-100). Median pemetrexed DI was 95% (IQR 75-100).

Primary endpoint – immune responses

Table 2A summarizes the primary endpoint, the generation of cellular and/or humoral immune responses specific for the tumor antigen (5T4) delivered by the vaccine. As 22/23

(96%) patients had an anti-5T4 response (either humoral or cellular), the study reached its primary endpoint. 17/23 (74%) of patients mounted humoral and 20/23 (87%) of patients developed cellular anti-5T4 immune responses, which were defined as doubling of immune responses from baseline. The longitudinal analysis of immune responses is shown on Table 2B and a time-related summary on Figure 2. The frequency of responders to 5T4 peaked at week 10, followed by a gradual decrease. This may have been due to the accumulated effects of 4 cycles of chemotherapy that started at 4-weeks and ended at 13-weeks. All three patients with sarcomatoid MPM mounted 5T4-specific T cell responses while antibody doubling was observed in 2/3 patients. In an independent preliminary study, involving 27 lung cancer and MPM patients who received pemetrexed-cisplatin chemotherapy without the vaccine, 5T4-specific antibody levels were measured before and after chemotherapy. Only one patient had a doubling of the 5T4-antibody level (data not shown), indicating that if changes in 5T4 antibody levels are observed in the trial, they will be due predominantly to the development of specific immune responses to the vaccine.

Clinical responses

For the 23 patients evaluable for clinical response by 26 weeks, 0/23 (0%) patients achieved a complete response, 4/23 (17%) a partial response, 16/23 (70%) a stable disease and 3/23 (13%) have progressed, giving an overall ORR of 17% (95% CI 5-39%) and disease control of 87% (95% CI 66-97%; Table 3). The median PFS was 6.8 months (95% CI 3.6-8.9) (Figure 3A). Median OS was 10.9 months (95% CI 8.1-23.5) (Figure 3B). The median OS for sarcomatoid patients was 6.6 months. The median length of follow-up for seven patients still alive was 24 months.

Adverse effects

Treatment-emergent adverse events experienced are shown in supplementary material Table S2. All 23 patients (100 %) had at least one grade 1-2 treatment-emergent AE. Grade 3-4 toxicities were seen in 11/23 (48%) patients: Grade 4 events included one patient with neutropenia and one with pleural effusion. Grade 3 events included three participants each with respiratory tract infection and thromboembolic events, and two participants with hypertension. There were also isolated grade 3 events: pyrexia, fall, cellulitis, phlebitis, and dyspnoea. There were no grade 5 toxicities. Grade 1-2 AE affecting more than 5 patients are summarized in Table S2.

Analysis of circulating immune cells

In this trial, 15 patients died within 14 months (Figure 3B) while the remaining 8 were still alive at 20 months. The frequency of patients alive in our cohort at 20 months (34.7%) correlates or is somewhat higher than the published frequency (24.8%)¹ and can be seen as the tail of the Kaplan Meyer curve (Fig 3B). We carried out a comparative analysis of FBC data and circulating immune cell phenotypes in patients with >20 months survival (8/23=34.7%) vs. those with <20 months (15/23=65.3%) survival. While there was no evidence that PFS was associated with baseline blood parameters (Table 4A) or that the >20m patients had any significantly different baseline FBC parameters, we found that NLR and MLR were significantly lower at week 4, when patients have only received 2 doses of vaccine but no chemotherapy, in the >20m group (Table 4B). Comparative analysis also indicated that more patients mounted T cell responses to 5T4 in the >20m group (Fig 4A) and T cell responses were generated with a broader 5T4 epitope specificity in the >20m compared to the <20m group (Fig 4B).

We also assessed the comparative frequencies of T cell, Treg cell, monocyte and MDSC subsets in baseline samples. As shown in Table 5, the immune parameters, found to be significantly different between the two groups were: higher CD8:CD4 ratios and lower naïve CD4 T cell frequencies in the >20m patients. There were no differences between the frequencies of either total or activated Treg cells between the groups, and the frequencies of main myeloid cell subsets were also comparable. These results indicate that elevated peripheral CD8+/CD4+ T cell ratio in MPM patients is a positive prognostic indicator in TroVax® treatment.

Tissue immunohistochemistry

19/23 pre-treatment FFPE samples were available for the immunohistochemical analysis of 5T4 expression and CD8+ T cell infiltration. 5T4 expression has been observed in all samples, although at varying degrees. In some cases, distinct expression was only observed on the tumor surface, while in others expression was observed in the tissue or in both sites (Fig 5A,B). There was no difference in the frequency of 5T4+ tumor cells in the tissue of long-term and short-term survivors, respectively ($32.7\pm 30.3\%$ vs. $46.5\pm 36.2\%$), however, expression was markedly lower in the sarcomatoid tumors ($2.33\pm 2.3\%$). CD8+ T cell infiltration was also studied. CD8+ cells were either scattered in the tissue or accumulated at the interface of malignant and normal tissues; in some cases both patterns were present (Fig 5A,C). Although there was a trend for higher CD8+ T cell infiltrate in the tissue of long-term vs. short-term survivors ($28.4\pm 33.3\%$ vs. $23.1\pm 24.8\%$), the difference was not significant. In contrast, CD8+ T cell infiltration was markedly lower in sarcomatoid tissues ($11.83\pm 15.8\%$). While the number of patients is insufficient for meaningful statistical analysis, we observed both 5T4 expression and CD8+ T cell infiltration at >10% of the tissue

in 50% of long-term survivors, while this was only true in 25% of short-term survivors and in none of the sarcomatoid tumors.

Discussion

In patients with previously untreated MPM, the SKOPOS trial reached its primary endpoint, showing that TroVax[®] in combination with pemetrexed-cisplatin chemotherapy results in 95.7% of patients developing immune responses against the 5T4 tumor antigen. In the independent preliminary study, pemetrexed-cisplatin chemotherapy alone did not result in immune responses to the 5T4 tumor antigen in 26/27 (96.3%) patients, so the immune responses seen in the trial are overwhelmingly likely to have been generated by the TroVax[®] vaccine.

The high rate of immune responses generated by the vaccine, despite being given in combination with potentially immunosuppressive chemotherapy, shows that MPM patients are capable of generating or reactivating tumor antigen specific T or B cell responses in first line treatment settings. The combination of TroVax[®] plus chemotherapy was well tolerated, with no significant additional toxicity seen as a result of the TroVax[®] vaccine. The proportion of patients getting one or more grade 3 or 4 toxicities was 48%, which compares to 62% reported in the chemotherapy-only arm of the MAPS trial. ²

With respect to clinical outcomes in SKOPOS, the median PFS of 6.8 months and median OS of 10.9 months are comparable to the median time to progression of 5.7 months and median OS of 12.6 months reported in the Pemetrexed-cisplatin arm of the Vogelzang trial, demonstrating that the combination with TroVax[®] has similar clinical activity. ¹ Importantly, we observed higher disease control (87% vs. 41.3%) than that reported in the Vogelzang trial and a greater proportion of patients achieved >20 months OS than those in the Vogelzang trial (34.7% vs. 24.8%). A feature of some contemporary immunotherapy trials in

solid tumors is the observation that the Kaplan-Meier survival curves do not start to separate from the standard chemotherapy arm in the initial months, and clinically meaningful differences only emerge when survival beyond the median timepoint is examined. For example, in the Checkmate 017 trial of the PD-L1 inhibitor nivolumab versus docetaxel chemotherapy in the advanced squamous cell lung cancer second line setting, median PFS was less than one month longer in the nivolumab-treated patients. However, this relatively modest increase in median PFS translated into a more impressive difference in one year PFS: 21% seen with nivolumab compared to 6% with chemotherapy.²⁴ Similar results were seen for OS in the sister Checkmate 057 trial in the second line advanced non-squamous lung cancer setting.²⁵ In a small, non-randomized trial such as SKOPOS it is not possible to draw any definitive conclusions about the significance of 34.7% long-term survivors, but one explanation is that an immune-mediated effect - as seen in the lung cancer Checkmate trials of nivolumab – may be driving a minority of patients to longer disease control and hence survival.

It is clear that not all tumors respond to immunotherapy. However, a strong correlation between tumor-infiltrating CD8+ T cells and favorable disease prognosis serves as a powerful indirect evidence for immune involvement in MPM.²⁶ Although our study was not statistically powered to analyze correlation between T cell infiltration and clinical benefit, we observed a trend for more CD8 infiltration in patients with longer survival. Despite these observations, to date, trials testing a variety of immunotherapeutic approaches in MPM have reported only modest clinical outcomes. For example, the anti CTLA-4 inhibitor Tremelimumab showed no improvement in overall survival compared to placebo as a later-line therapy in patients with pleural and peritoneal malignant mesothelioma.²⁷ In the single

arm Keynote 028 trial, the anti PD-L1 antibody Pembrolizumab reported more encouraging results, with a median PFS of 5.8 months and a disease control rate of 76% in MPM patients previously treated with chemotherapy.²⁸

The biggest limitation of the SKOPOS trial is the single arm design with no control group; no definitive conclusions can be drawn when comparing the clinical outcomes from SKOPOS with published historical controls of patients treated with chemotherapy alone. However, we did show that pemetrexed-cisplatin chemotherapy alone elicited a 5T4 immune response (doubling of antibody response) in only 1/29 (3.4%) lung cancer and mesothelioma patients so we are confident that the immunological activity seen in the SKOPOS trial is predominantly due to the TroVax[®] vaccine. Another observation we made, albeit only in 3/23 of samples, that patients with sarcomatoid MPM were just as able to mount systemic 5T4 immune responses following the vaccine treatment as the cohort with epithelioid tumors. However, the pre-treatment tissue samples revealed low antigen expression and weak T cell infiltration into the tissue, indicating more powerful immunosuppression or at least lack of immune support in the tumor microenvironment of these patients, potentially contributing to the more rapid disease progression.

Predictive biomarkers are important in providing bespoke cancer treatment for patients. The results from SKOPOS suggest that inducing immune responses against the 5T4 tumor antigen alone does not select patients more likely to have a longer survival - almost all patients in the study developed such an immune response. We have carried out retrospective data-analysis to look for possible differences between those surviving >20m vs. <20m. We observed a high CD8:CD4 T cell ratio in long-term survivors, consistent with

the positive prognostic significance of CD8+ T cells in MPM patients.²⁶ We also identified elevated levels of CD4+ naïve T cell in the short-term survivor group of patients. This may indicate that MHC Class II antigen presentation was suboptimal in these patients. Although naïve T cells exposed to high levels of TGF β in the plasma are easily hijacked to become Tregs²⁹, we did not observe significant differences in Treg frequencies in the circulation of these patients. However, we have not studied the intratumoral frequencies of Treg that have a known prognostic significance.⁷

Again, no differences were observed in classical monocyte frequencies (CD14+ HLA-DR+), or in the frequencies of cells expressing M-MDSC, G-MDSC or E-MDSC markers.³⁰

Given the disappointing results of numerous cancer vaccine trials, it seems unlikely that a vaccine alone will have a significant impact on solid tumors, although this type of treatment may be beneficial for some patients. While the combination of a vaccine and chemotherapy may not be optimal, as chemotherapy may damage expanding T cell populations, the combination of a vaccine and a checkpoint inhibitor may lead to synergistic results. Checkpoint inhibitors may also overcome potentially inhibitory signalling pathways that switch off T-cells and dampen the immune response to the tumor antigen. We believe the SKOPOS trial participants are representative of a significant proportion of newly diagnosed MPM patients in the UK, and the results of any subsequent clinical trials using combination treatment schedules would be broadly applicable.

Materials and methods

Study design and participants

We undertook an open-label, single-arm phase II trial (SKOPOS) at Velindre Cancer Centre, Cardiff, UK. Patients with locally advanced or metastatic, histologically or cytologically confirmed, MPM were potentially eligible for the trial. Inclusion criteria included age ≥ 18 years, World Health Organization (WHO) performance status (PS) 0-1 and an estimated life expectancy of at least six months. Hematological inclusion criteria were hemoglobin ≥ 10 g/dL, total white cell count $\geq 3 \times 10^9$ /L, neutrophil count $> 1.5 \times 10^9$ /L, lymphocyte count $\geq 0.8 \times 10^9$ /L, monocyte count $< 1 \times 10^9$ /L and platelet count $100-500 \times 10^9$ /L. Patients also had to have adequate renal and liver function.

Exclusion criteria included major surgery, serious infection or radiotherapy (superficial radiotherapy to chest wall sites was permitted) in the four weeks prior to trial entry, previous TroVax[®] or chemotherapy treatment, cerebral metastases or history of allergic response to previous vaccinia vaccinations.

All patients provided written informed consent before enrolment. Approval from a UK Research Ethics Committee, (Ref: GTAC174), and Medicines and Health Care Products regulatory Committee (EudraCT2010-023230-22) was obtained. The trial was coordinated by the Wales Cancer Trials Unit at Cardiff University and sponsored by Velindre NHS Trust. The trial was conducted in accordance with the principles of the Declaration of Helsinki and the ICH Good Clinical Practice, and registered with ClinicalTrials.gov (NCT01569919).

In order to establish that chemotherapy alone does not elicit an immune response, an independent pre-study was also conducted, in which blood samples from 27 lung cancer and MPM patients were tested for antibody responses to the 5T4 antigen before and after pemetrexed-cisplatin chemotherapy.

Procedures

As summarized on the CONSORT flow diagram (Figure 1), eligible patients received TroVax® by intramuscular injection at a dose of 1×10^9 TCID₅₀/mL in 1mL, given on Day 1 or 2 of weeks 1, 3, and then every three weeks to week 24. Patients also received 4 cycles of Pemetrexed (500 mg/m² over 10 min) and Cisplatin (75mg/m² over 1 h), given on day 3 or 4, from week 4. Folic acid, vitamin B12 and corticosteroids were administered according to protocol.

A baseline CT scan of the chest and upper abdomen had to be undertaken with documentation of known measurable or evaluable disease parameters using the modified Response Evaluation Criteria in Solid Tumors (RECIST) criteria for mesothelioma.²⁰ WHO PS and toxicity were collected at baseline according to the Common Terminology Criteria for Adverse Events (v4.02). Tumor response was assessed with CT at week 16, 26, 39 and 52 weeks and with RECIST V1.1. Toxicity assessments were carried out at week 4, 7, 10, 13, 16, 26 and 34 with serious adverse events being collected in real time.

Blood sample collection and processing

Venous blood samples were collected into EDTA vacutainers (Greiner #455036) at six time points for immunological testing: baseline (80 mL), week 4, 7, 10, 13, 26 and 34 (50 mL each). PBMC were isolated by density gradient centrifugation on Histopaque (Sigma #H8889), within 30 min of collection. The plasma was frozen in 10 cryovials (Sarstedt #72.377) in 0.5 mL aliquots at -80°C. PBMC were frozen in freezing media comprising 10% DMSO (Sigma #D2650), 20% FBS (Gibco, #26140-079 lot #1233760; batch-tested for low T cell mitogenicity) in RPMI 1640 media (Sigma #BE12-167F), supplemented with 2 mM L-

Glutamine (Lonza #BE17-605E), 100 U/ml penicillin/100 µg/mL streptomycin (Lonza #BE17-603E), 25 mM HEPES (Sigma #H0887) and sodium pyruvate (Sigma #S8636). PBMC were frozen as one 2.6×10^7 vial, with the remaining cells in around 1.3×10^7 cells per vials at -80°C in a CoolCell container (VWR) overnight, before transferring to vapor phase of liquid nitrogen. T cell assays were carried out when all longitudinal samples were obtained.

Measurement of 5T4-specific T cell responses

The intracellular cytokine staining protocol used to assess 5T4 specific T cell responses was optimized prior to the trial²¹ (and unpublished work). 5T4 peptides (42 in total) spanning the entire 5T4 protein sequence (15-mers, overlapping by 5 amino acids) were synthesised by ProImmune at >85% purity. They were reconstituted in DMSO at a concentration of 10 mg/mL and stored in -20°C . Peptides were pooled into 4 separate groups (peptide pools 1-10, 11-20, 21-31 and 32-42) prior to use. Pools of viral peptides (Class I and II epitopes) representing cytomegalovirus, Epstein-Barr and influenza virus (CTL #CTL-CEF-001 and #CTL-CEFT-001) epitopes were used as positive and DMSO as negative control. Frozen PBMC samples were thawed and viability determined with trypan blue (Sigma #T8154) staining. Any samples with less than 85% viability were not used. PBMC (8×10^5 - 10^6) were seeded in 48-well plates (Greiner #677180) in 1 mL supplemented RPMI containing 10% FBS. 5T4 peptide pools (20 µg/mL), viral peptide pool (5 µg/mL) and DMSO (1 µL/mL; negative control) were added to relevant wells in addition to 1000 U/mL IFN α (R&D #11101-2), 20 ng/mL IL-1 β and 500 U/mL IL-6 (Peprotech #200-01B and 200-06, respectively). Cells were cultured for 6 days then restimulated using autologous B lymphoblastoid cell lines (BLCL) at 20:1 ratio (5×10^4 BLCL to 1×10^6 PBMC). BLCL were loaded with 10 µg/mL of each 5T4 peptide pool, or 2.5 µg/mL viral peptide pool or volume equivalent of DMSO, for 4 h at 37°C .

BLCL were co-incubated with the relevant PBMC in FACS tubes (VWR #352054) for 1 h at 37°C prior to adding Golgi Plug (0.5 µL; BD #555029) and Golgi Stop (0.35 µL; BD #554724) in a final volume of 500 µL followed by a further 12-13 h incubation. Cells were washed in PBS (Lonza #BE17-512F) and labelled with 0.5 µL LIVE/DEAD e-Fluor 780 fixable dye (Affymetrix eBioscience #65-0865-14) in 500 µL at 4°C for 30 min. Cells were fixed in 100 µL Affymetrix eBioscience fixation buffer (#00-8222-49) for 15 min at room temperature. Cells were then washed once in PBS, permeabilized in 100 µL 1x permeabilization buffer (Affymetrix eBioscience #00-8333-56) and labelled with 2.5 µL each of CD3 PE-Cy7 (#25-0038), CD4 APC (#17-0049), CD8a PerCP-Cy5.5 (#45-0088), IFN γ PE (#12-7319), TNF α e-fluor 450 (#48-7349) and IL-2 FITC (# 11-7029; all from Affymetrix eBiosciences) antibodies for 40 min at room temperature in the dark. Samples were then washed in staining buffer, resuspended in 230 µL staining buffer and run on a BD FACSVerser flow cytometer which was normalized daily with CS&T bead tracking. Compensation controls were established with cells labelled with one antibody at a time. Data were acquired with BD FACSuite software. Gating for cytokine-producing T cells was carried out by following the Cancer Immunotherapy Consortium's guidelines.²² This was based on an international assay/gating harmonization exercise in which our laboratory took part (Supplementary Figure 1). All results were audited and raw data can be provided on request.

Measurement of Antibody Responses

5T4- and MVA-specific antibody responses were determined from longitudinal plasma samples using a validated semi-quantitative Enzyme linked immunosorbant assay (ELISA).²³

Immune cell phenotyping

PBMC (2×10^5 /tube) were labelled in 100 μ L staining buffer for T cells, monocytes, myeloid-derived suppressor cells (MDSC) and Treg cells using antibodies detailed in Table S1. Cells were labelled for surface markers for 40 min on ice. Cells in the Treg panel were then fixed, permeabilized and further labelled with Foxp3 FITC and Ki67 APC, according to the manufacturer's instructions. All cells were run on a BD FACSVerser flow cytometer and analysed with Diva 8 software.

Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) sections were prepared. Antigen retrieval was performed on the Dako Omnis platform by heating slides in retrieval solution at 97° C for 30 min then at room temperature and washing in distilled water. Sections for 5T4 (R&D Systems # MAB 4975) underwent high pH 8.5 EDTA antigen retrieval. Sections for CD8 (Dako Agilent mouse monoclonal clone C8/144B) had pH 6.0 citrate antigen retrieval. 5T4 antibody was used at 1:75 dilution of the stock of 0.5 mg/ml. CD8 antibody was in a ready to use formulation. Both 5T4 and CD8 had primary antibody incubations at 37°C (5T4 for 30 min, CD8 for 20 min) with detection by the DAB Omnis kit. Slides were analyzed on a Nikon eclipse E600 light microscope by an experienced mesothelioma pathologist (RLA). A semi-quantitative evaluation of 5T4 staining was made noting either no, mild, moderate, intense membranous expression of surface and deep tumor tissue. A semi-quantitative evaluation of cytotoxic CD8 T-cells was made noting a nil, mild, moderate or plentiful response within tumor or at the tumor-stromal interface. Percentages and patterns of expression were recorded.

Outcomes

The primary outcome measure was pre-defined as a doubling of anti-5T4 immune responses compared to those at baseline at any of the six time points. Secondary outcome measures included the safety and tolerability of TroVax® in combination with pemetrexed/cisplatin, progression-free survival (PFS), objective response rate (ORR), and OS. The study also investigated the relationship between immune and clinical responses, the utility of baseline platelet count, monocyte count, hemoglobin levels, neutrophil:lymphocyte ratios (NLR) and monocyte:lymphocyte ratios (MLR) as predictors of treatment benefit. The latter two were also analyzed at the 4-week time point.

Statistical analysis

We used a Fleming's single arm design with the outcome measure of immune response to the 5T4 antigen. If less than 40% of patients demonstrated an increased response from baseline, then we would not pursue further research. If an increased response was seen in 64% or more of patients then this would justify further research in the vaccine in patients with MPM. Setting $\alpha=0.05$ (1-sided) and 80% power, 26 participants were required. If a doubling of 5T4 was seen in at least 16 patients, the null hypothesis that the vaccine does not elicit an immune response could be rejected. A per protocol analysis (PPA) was used where a patient had to receive at least the first three TroVax injections and the first cycle of chemotherapy (at full or reduced dose). PFS was calculated from the day of trial entry to the date of first clinical evidence of local progression or death (of any cause). Patients progression-free and alive were censored at the time last seen. Extended survival time was collected from the site beyond the end of the one-year follow-up after obtaining ethical approval. OS was calculated from the date of trial entry to the day of death (any cause). Those still alive were censored at the time last seen. PFS and OS were presented in time to

event format using Kaplan-Meier curves with median time and their corresponding 95% CIs. In a planned exploratory analysis a Cox proportional hazard model was used to explore whether baseline platelet count, baseline monocyte count and baseline hemoglobin predict time to progression. The univariate hazard ratios for each predictor are presented with their corresponding p-value. A logistic regression model was used to explore the effect of hemoglobin, hematocrit, soluble mesothelin and baseline 5T4 antibody level on the post-treatment 5T4 antibody response. The primary endpoint and other secondary categorical endpoints (best response by 26 weeks) were presented as % and 95% CIs. Percentage dose intensity (DI) was calculated as total dose received in mg per m² (m²)/total expected dose x100. All analysis used STATA 14.0.

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Figure legends

Figure 1. CONSORT Flow diagram of patients allocated for the SKOPOS trial.

Figure 2. Summary of any 5T4-specific immune response in a longitudinal study. The bars represent the proportions (%) of patients whose immune response was ≥ 2 -fold to any 5T4 peptide group compared to their immune responses at baseline. The six time points are indicated on the X-axis.

Figure 3. Kaplan-Meier curves for (A) Progression-free survival (PFS), (B) Overall survival (OS).

Figure 4. T cell responses to 5T4 peptides by long term vs. short term survivors. (A) The proportions of patients who generated CD4+ or CD8+ or both types of T cell responses to the 5T4 antigen. (B) The average frequencies of peptide groups that T cell responses were generated against at different time points during the trial. (A,B) <20 mo (black) represents patients with less than 20 months, while >20 mo (gray) those with more than 20 months OS.

Figure 5. Immunohistochemistry of 5T4 expression and CD8+ T cell infiltration in pre-treatment tissue. (A) Expression levels of 5T4 (i) and CD8 (ii) in the specimens of long term survivors (>20 mo; ▼), short-term survivors (<20 mo; ○) or sarcomatoid patients (sarc; ◇). Each symbols represents a different patient. The vertical bars represent mean of expression. (iii) The combination of results from (i) and (ii). Reference lines represent 10% of expression. (B) Representative examples of 5T4 expression in epithelioid tissue. (i) High tissue expression, (ii) high tumor tissue and surface expression, (iii) low tissue expression. (C)

representative examples of CD8+ T cell infiltration. (i) high frequency scattered tissue infiltration, (ii) accumulation at the tumour margin, (iii) low level of tissue expression. Magnifications are 200x, except for Cii (100x).

Tables

Table 1: Participant baseline characteristics

Characteristics	Results (N=23)
Age ¹	66 (61-70)
Sex	
Male	20 (87.0%)
Female	3 (13.0%)
WHO performance status	
0	12 (52.2%)
1	11 (47.8%)
Mesothelioma type	
Epithelioid	20 (87.0%)
Sarcomatoid	3 (13.0%)
Mesothelioma stage	
Stage II	8 (34.8%)
Stage III	13 (56.5%)
Stage IV	2 (8.7%)
Platelets ¹	351 (250-407)
Monocytes ¹	0.6 (0.4-0.8)
Hemoglobin ¹	13.8 (12.7-14.7)

1 – Median (IQR)

Table 2: Immune responses**A. Summary of overall immune responses – primary endpoint**

Measurements	Results (N=23)
Humoral 5T4 overall response	17 (73.9%)*
Cellular 5T4 overall response	20 (87.0%)
Any overall response (humoral or cellular 5T4 immune response)	22 (95.7%)

*Number of patients responded (%)

B. Detailed immune responses – time kinetics

Immune parameters	n/N (%) patients					
	Week 4	Week 7	Week 10	Week 13	Week 26	Week 34
CD4 IFN γ	4/21 (19.05%)	12/22 (54.55%)	11/22 (50.00%)	7/18 (38.89%)	4/15 (26.67%)	4/11 (36.36%)
CD8 IFN γ	1/21 (4.76%)	5/22 (22.73%)	5/22 (22.73%)	5/18 (27.78%)	4/15 (26.67%)	2/11 (18.18%)
CD4 TNF α	2/21 (9.52%)	3/22 (13.64%)	5/22 (22.73%)	1/18 (5.56%)	4/15 (26.67%)	3/11 (27.27%)
CD8 TNF α	0/21 (0%)	0/21 (0%)	2/22 (9.09%)	2/18 (11.11%)	2/15 (13.33%)	1/11 (9.09%)
CD4 IL-2	3/21 (14.29%)	3/22 (13.64%)	5/22 (22.73%)	3/18 (16.67%)	6/15 (40.00%)	2/11 (18.18%)
CD8 IL-2	3/21 (14.29%)	2/22 (9.09%)	3/22 (13.64%)	3/18 (16.67%)	1/15 (6.67%)	1/11 (9.09%)
Humoral 5T4	3/23 (13.04%)	12/23 (52.17%)	10/22 (45.45%)	9/19 (47.37%)	10/16 (62.50%)	5/12 (41.67%)
Cellular 5T4	9/21 (42.86%)	13/22 (59.09%)	14/22 (63.64%)	11/18 (61.11%)	9/15 (60.00%)	5/11 (45.45%)
Any (cellular or humoral 5T4)	11/23 (47.83%)	19/23 (82.61%)	20/22 (90.91%)	15/19 (78.95%)	13/16 (81.25%)	9/12 (75.00%)
Humoral MVA	22/23 (95.65%)	23/23 (100.00%)	22/22 (100.00%)	18/19 (94.74%)	16/16 (100.00%)	12/12 (100.00%)

Table 3. Clinical responses

Best response (pleural disease, target or non-target tumor) ¹	
Complete response	0/23 (0%)
Partial response	4/23 (17.4%)
Stable disease	16/23 (69.6%)
Progressive disease	2/23 (8.7%)
Unknown ²	1/23 (4.3%)
ORR (CR+PR) ¹	4 /23 (17.4%)
Disease Control Rate (CR+PR+SD) ¹	20/23 (87.0%)
Median PFS, months ³	6.8 (3.6-8.9)
Rate of PFS at 6 months ⁴	60.6% (37.8-77.2%)
Rate of PFS at 12 months ⁴	23.3% (8.5-42.2%)
Median OS, months ³	10.9 (8.1-23.5)
Rate OS at 6 months ⁴	82.6% (60.1-93.1%)
Rate OS at 12 months ⁴	43.5% (23.3-62.1%)
Follow-up, months (alive) ³	24.0 (19.3-NR)

1 – Number of patients n/23 (% responders)

2 – RECIST data is not available for one patient

3 – months (95% CI)

4 – % of patients (95% CI)

NR –Not reached

Table 4A. Analysis of full blood count data

Variable	Hazard Ratio	Lower bound of 95% confidence interval	Upper bound of 95% confidence interval	p-value
Platelets	0.998	0.993	1.003	0.43
Monocytes	0.693	0.073	6.536	0.75
Haemoglobin	1.217	0.897	1.651	0.21
Haematocrit*10	1.903	0.628	5.767	0.26
Mesothelin	0.937	0.857	1.025	0.16
Humoral 5T4	1.007	0.975	1.040	0.67

Exploratory univariate analysis of baseline blood parameters and PFS

Table 4B. Retrospective analysis of FBC

	>20 weeks ^a	<20 weeks ^b	p-value ^c
Monocytes (B)	0.587±0.176	0.707±0.19	0.0833
Platelets (B)	343.8±126	343.5±113	0.389
Haematocrit (B)	0.406±0.047	0.413±0.003	0.348
NLR^d (B)	3.05±1.61	3.268±1.55	0.378
NLR (W4)	2.401±1.25	3.321±0.98	0.041(*)
MLR^e (B)	0.324±0.099	0.415±0.173	0.0747
MLR (W4)	0.268±0.111	0.415±0.171	0.021(*)

(a) Patients who survived >20 weeks

(b) Patients who died before 20 weeks

(c) P value by one sided t-test

(d) Neutrophil:lymphocyte ratio

(e) Monocyte:lymphocyte ratio

B: baseline; W4: 4 weeks after entering trial

Table 5. Phenotypic analysis of circulating immune cells at baseline

T cells	<20 months	>20 months	p-value
CD8:CD4 Ratio	0.571±0.079	0.995±0.389	0.035 (*)
CD4+CD27+CD45RA- (CM)	34.07±3.374	38.59±6.430	0.249
CD4+CD27+CD45RA+ (N)	59.38±3.957	42.93±7.629	0.023 (*)
CD4+CD27-CD45RA- (EM)	4.258±0.653	6.758±2.375	0.104
CD4+CD27-CD45RA+ (TEM)	2.292±0.948	11.72±9.717	0.098
CD8+CD27+CD45RA- (CM)	30.13±3.844	22.41±5.384	0.127
CD8+CD27+CD45RA+ (N)	42.10±4.734	44.98±8.891	0.379
CD8+CD27-CD45RA- (EM)	5.408±1.098	3.919±1.188	0.201
CD8+CD27-CD45RA+ (TEM)	22.37±5.650	28.72±9.866	0.276
Treg cells			
Treg (CD3+CD4+CD25+Foxp3+)	5.052±0.368	5.938±1.077	0.174
CTLA-4+ of Treg	5.848±0.623	6.730±2.291	0.319
Ki67+ of Treg	22.45±2.116	20.26±2.049	0.256
Myeloid cells			
(DC) CD14- HLA-DR+	5.018±0.470	6.269±1.149	0.123
(MPh) CD14+ HLA-DR+	8.682±1.044	7.263±1.070	0.198
PDL-1+ MPh	11.10±2.085	12.30±3.137	0.721
CD200R+ PDL-1+ MPh	8.613±1.780	6.422±1.898	0.222
MDSC			
M-MDSC (CD14+ HLA-DR- CD11b+ CD15-)	4.468±3.079	3.317±1.584	0.186
G-MDSC (CD14- CD33- CD15- CD11b+)	0.028±0.011	0.276±0.555	0.186
E-MDSC (Lineage- CD15- CD11b+ CD33+)	0.079±0.098	0.502±1.01	0.058

C: central memory; N: naïve; EM: effector memory; TEM; terminally differentiated effector memory. MPh; macrophage.

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Contributors

JL, GG, AC, RH and ZT designed and developed the study and wrote and reviewed the protocol. JL played a part in day-to-day running of the study. LK and AC did statistical analyses. JL reviewed serious adverse events during the study. SA-T and ZT undertook the laboratory work. **RLA directed the IHC staining and conducted the tissue analysis.** All authors contributed to the writing of the manuscript.

Role of the funding source

The funder and sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. AC and LK had full access to all the data in the study. All authors had final responsibility for the decision to submit for publication.

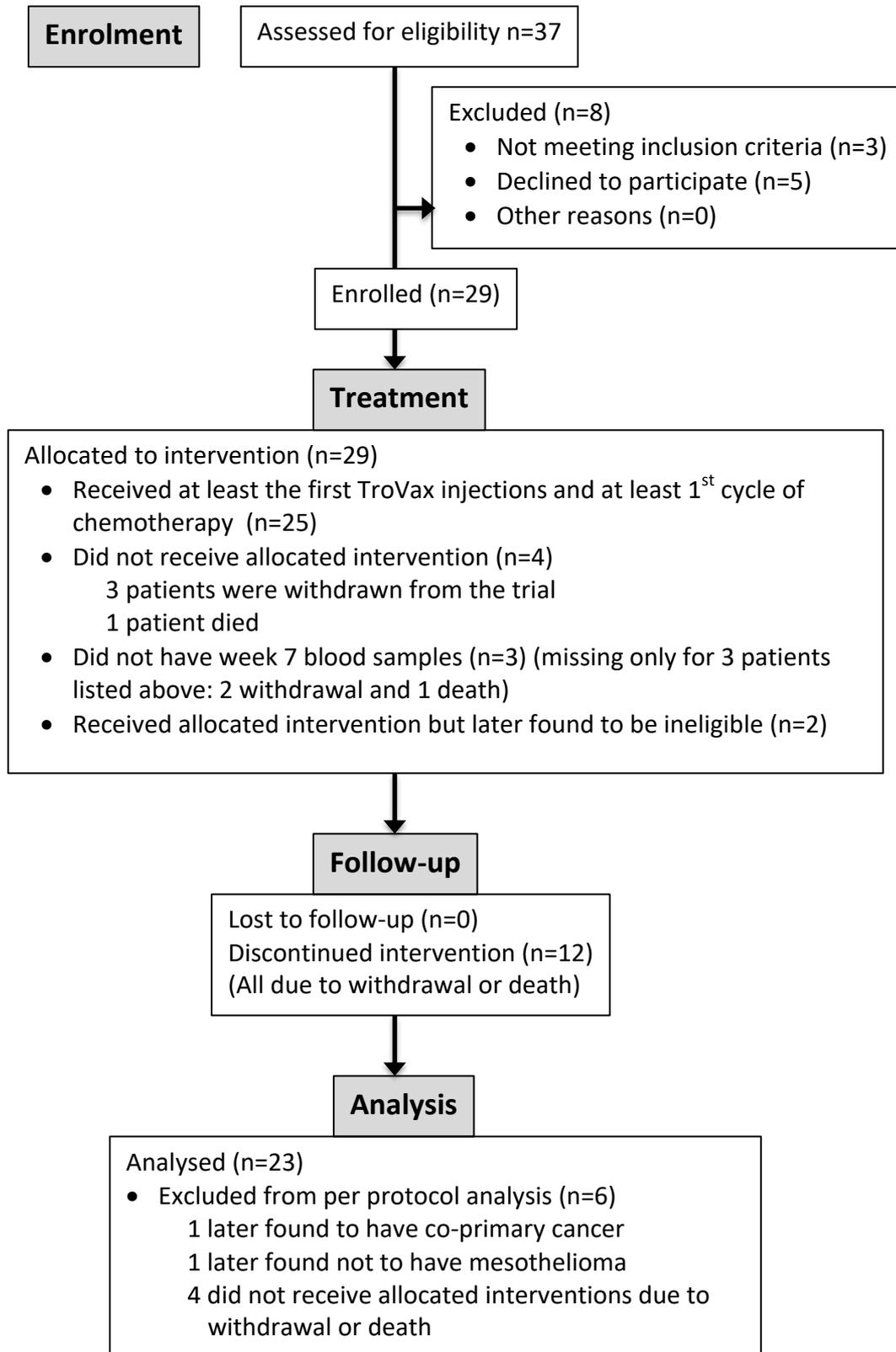


Figure 2

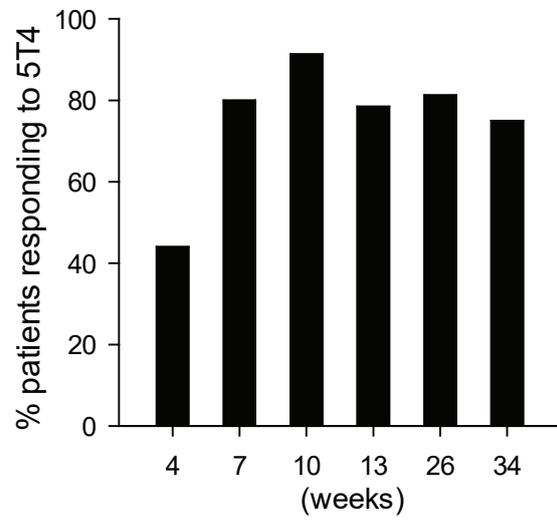
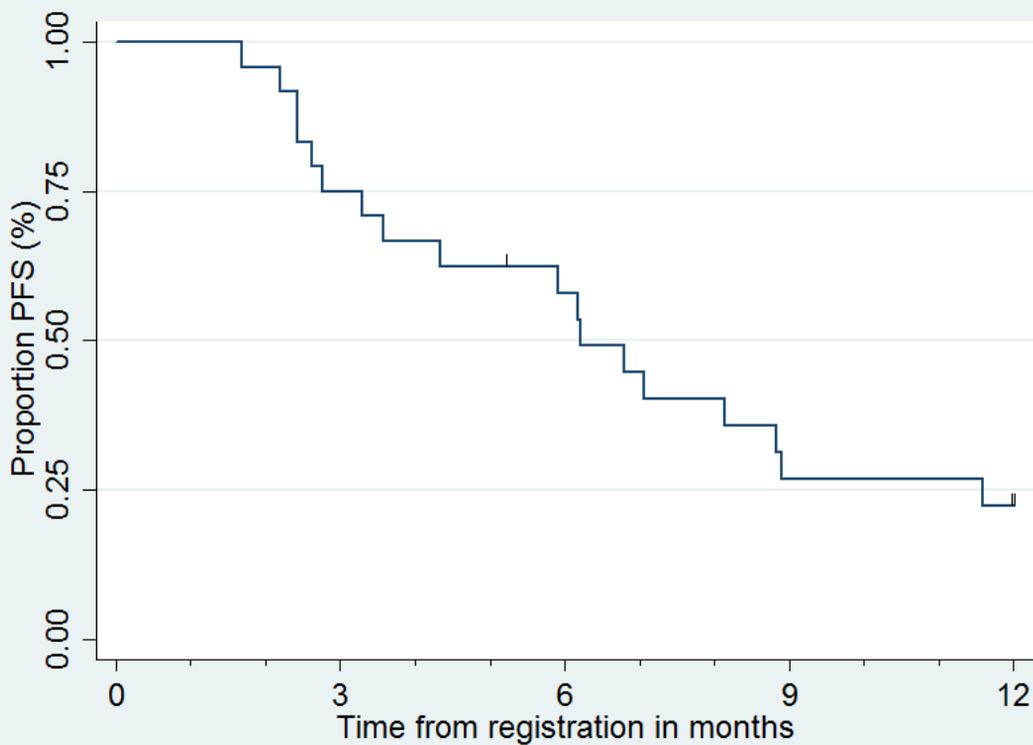


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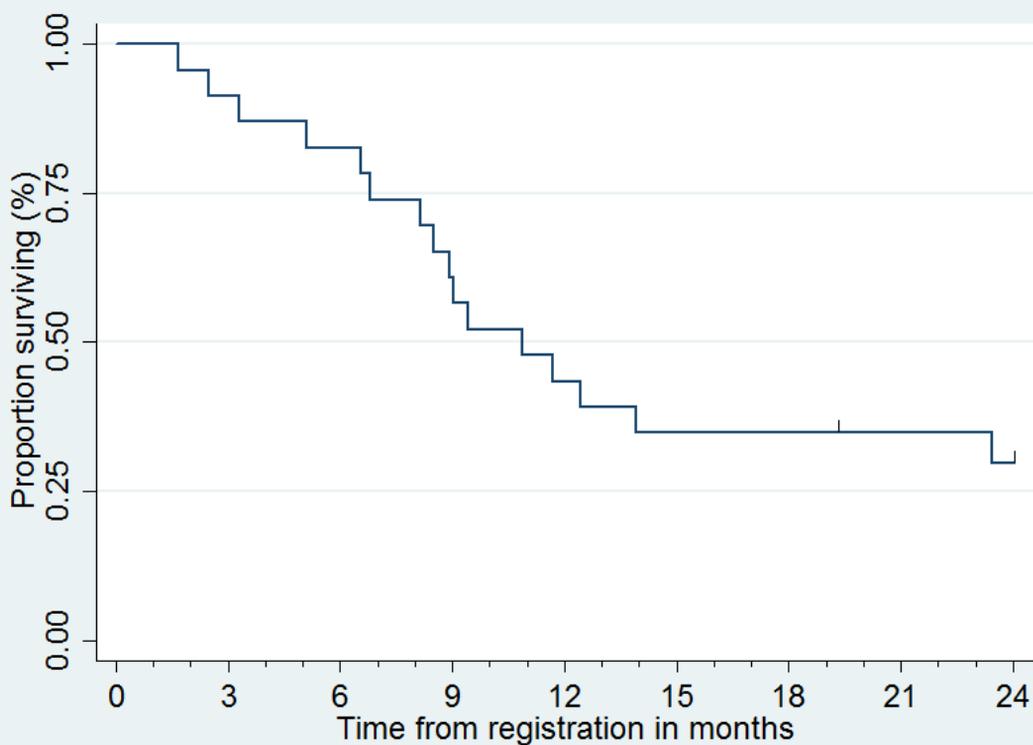
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Number at risk

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B



Number at risk

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Figure 4.

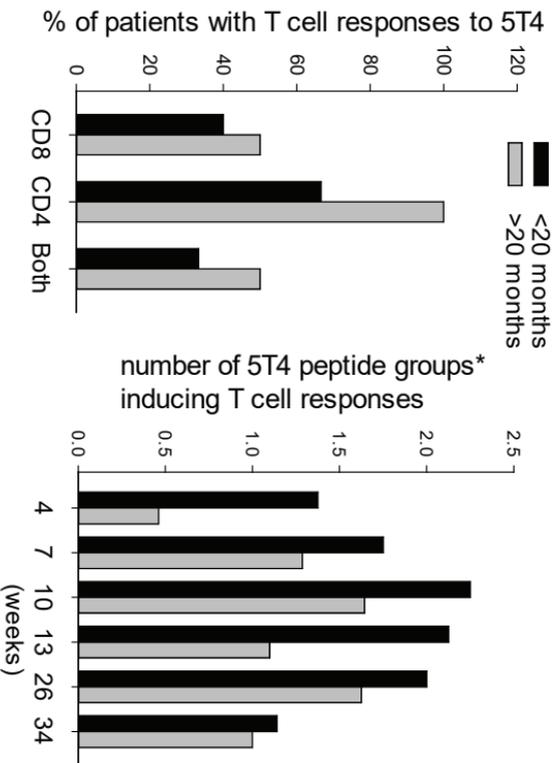


Figure 5

