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MVA-5T4 immunotherapy (TroVax) and low-dose cyclophosphamide for advanced colorectal cancer (TaCTiCC): an open-label, randomized phase 1/2 clinical trial

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Keywords: Cyclophosphamide, TroVax, colorectal cancer, T-cells.

Abbreviations Used: CPM, cyclophosphamide; CRC, colorectal cancer; CT, computerized tomography; ELISpot, enzyme-linked immunoSpot; FACS, fluorescence-activated cell sorting; Foxp3, Forkhead Box p3; IFN-γ, Interferon-γ; Mo, Months; MVA, modified vaccinia Ankara; OS, overall survival; PBMC, peripheral blood mononuclear cell; PFS, progression-free survival; PPD, purified protein derivative; RU, relative units; SFC, spot-forming cell; TAA, tumor-associated antigen; TaCTiCC, TroVax and Cyclophosphamide Treatment in Colorectal Cancer; TD, treatment day; Treg, regulatory T-cell.

Key Points

Question

Does low-dose cyclophosphamide (CPM) ±MVA-5T4 (TroVax) enhance anti-tumor immunity in metastatic colorectal cancer (mCRC) patients in a randomized controlled trial?

Findings

All three treatment regimens of CPM-only, TroVax-only and combination demonstrated encouraging anti-tumor immunological responses, prolonging survival with no safety concerns. The addition of CPM made no difference to the number of patients responding to TroVax; however, this is the first randomized trial demonstrating improved survival outcomes of mCRC patients with low-dose CPM.

Meaning

These data support the importance of well-targeted anti-tumor immune responses, show the safety and anti-tumor activity of both low-dose CPM and TroVax, and support further investigation in mCRC.

Abstract

Importance The success of immunotherapy with checkpoint inhibitors is not replicated in most cases of colorectal cancer, so different strategies are urgently required. The oncofetal antigen 5T4 is expressed in >90% of metastatic colorectal cancer (mCRC). Preliminary data using a 5T4-expressing vaccine (TroVax) in mCRC demonstrated it safely induced serological and T-cell responses.

Objective TroVax was combined with metronomic low dose cyclophosphamide (CPM), as a regulatory T-cell (Treg)-depleting agent to determine whether anti-tumor immunity could be boosted, in an open randomized study in mCRC.

Design, Setting and Participants Between July 9, 2012, and February 8, 2016, fifty-five inoperable mCRC patients with prior stable disease following standard chemotherapy, were enrolled at a single-center and randomized to groups 1: no treatment (n=9); 2: CPM (n=9); 3: TroVax (n=19); 4: combination of TroVax and CPM (n=18).

Interventions Patients randomized to a CPM group received 50mg b.i.d. on treatment days 1-7 and 15-21. Patients randomized to a TroVax group received at a dose of $1x10^9$ $TCID_{50}$ as an intramuscular injection at treatment days 22, 36, 50, 64, 78 and 106.

Main Outcomes and Measures The pre-defined primary endpoint was the magnitude of anti-5T4 immune responses (5T4-specific T-cells and antibodies) generated at treatment week 7. Secondary endpoints included analysis of the kinetics of anti-5T4 responses, progression-free survival (PFS) and overall survival (OS).

Results 5T4-specific immune responses were significantly increased in groups 3 (p=.02) and 4 (p=.002). CPM depleted Tregs in 24/27 patients, independently prolonging PFS (5.0 vs. 2.5 months, HR=0.48, 95%-CI 0.21-1.11, p=.09). TroVax doubled baseline anti-5T4 responses in 16/35 patients, resulting in significantly prolonged PFS (5.6 vs. 2.4 months, HR 0.21, 95%-CI 0.090-0.47, p<.001) and OS (20.0 vs. 10.3 months, HR=0.32, 95%-CI 0.14-0.74, p=.008). No grade 3/4 adverse events were observed.

Conclusion and Relevance This is the first randomized immunotherapy study demonstrating a significant survival benefit in mCRC. Prior depletion of Tregs by CPM does not boost immune responses generated to TroVax vaccination, however both CPM and TroVax independently induced beneficial anti-tumor immune responses resulting in prolonged survival without toxicity. Larger clinical trials are planned to further validate these data.

Trial Registration EudraCT: 2010-024380-41. ISRCTN registry: ISRCTN54669986.

Introduction

Colorectal cancer is the second leading cause of death from cancer.[1] Although early stages are often cured by surgical resection, the prognosis for patients with metastatic colorectal cancer (mCRC) is very poor, with a 5-year survival rate of 7%.[2]

There is a clear unmet need for improved therapies; whilst immunotherapy has been at the forefront of recent advances, results in CRC have been disappointing. Over 96% of mCRC patients have microsatellite stable tumors[3] that do not respond to current immunotherapies, possibly due to decreased incidence of neo-antigens.[4-6] We hypothesized that in these patients, a well-targeted immune response against an upregulated tumor antigen, with minimal expression on healthy background tissues, represents a potentially more powerful therapy. One candidate is 5T4, a trophoblast glycoprotein with restricted expression to several human adenocarcinomas including >90% of CRCs.[7,8] Previously, we demonstrated that 5T4-specific IFN-γ^+ T-cell responses correlate with tumor stage providing protection against metastasis.[9,10] Here, we sought to improve 5T4 immune responses in mCRC patients by vaccinating with an immunogenic, non-replicating modified vaccinia Ankara (MVA) vector encoding the 5T4 antigen (TroVax). This vaccine has demonstrated efficacy in pre-clinical models of colon cancer via the induction of humoral anti-5T4 responses.[11] Early indications in mCRC patients demonstrated an excellent safety profile, with the induction of anti-5T4 responses correlating with disease control, thus warranting further studies in randomized trials.[12]

Previous attempts at vaccination strategies targeting upregulated tumor antigens have been largely unsuccessful for many tumor types; however a recent trial of MVA-MUC in advanced NSCLC demonstrated profound improvement to PFS.[13] Such vaccines work by inducing the intracellular expression of their respective transgene allowing the tumor antigen to be processed by MHC class-I and –II pathways. Given that activation of the

adaptive immune response may concurrently stimulate tumor-specific regulatory T-cells (Tregs), we also sought to test the hypothesis that the effectiveness of cancer vaccines is improved by prior administration of a Treg depleting agent. In low-doses, CPM has demonstrated numerous immune potentiating effects, most notably the depletion and reduced functionality of Tregs.[14,15] However, to date, low-dose metronomic CPM has not been evaluated in a randomized-controlled setting for cancer.

The trial reported herein is a randomized phase 1/2 trial in patients with inoperable, mCRC, aiming to assess the effectiveness of CPM to boost the immunotherapeutic potential of TroVax. We present the final analyses of primary and secondary endpoints, including an assessment of how anti-5T4 immune responses and Treg depletion associates with patient survival.

Methods

Study design and participants

This open-label study was performed in a single-centre in the Clinical Research Facility, University Hospital of Wales, UK. Patients were evaluated for recruitment at Velindre NHS Trust and the South West Wales Cancer Centre, UK. Patients were eligible if they had inoperable stage-IV CRC, providing they had evidence of responding or stable disease within 4-weeks of trial entry. Previous findings indicate that patients receiving palliative chemotherapy can safely be given protracted breaks, i.e. chemotherapy "holidays", with no evidence of a worsening of their outcome.[16] However, patients with elevated platelet counts (>400,000/μl) did not tolerate chemotherapy-free intervals and were excluded from this study. Additional exclusion criteria included haemoglobin <11g/dL, monocyte count >80,000/μl, completion of first-line chemotherapy <2 weeks from start of treatment, clinically apparent autoimmune disease, or those receiving immunosuppressants. Key inclusion criteria included WHO performance status 0-2, lymphocyte count ≥500/μl and neutrophil count >1200/μl.

All patients gave written, informed consent personally prior to trial inclusion. The Gene Therapy Advisory Committee (GTAC175) and the Cardiff and Vale Ethics committee approved the study. Trial authorization was granted from the Medicines and Healthcare products Regulatory Authority. This trial is registered with EudraCT (2010-024380-41), the ISRCTN registry (ISRCTN54669986) and was conducted in compliance to ICH-GCP regulatory requirements.

Randomization and masking

The trial was based on a 2x2 factorial design. Patients were randomized 1:1 between receiving CPM and not, and 2:1 between receiving TroVax and not, giving rise to four

treatment arms: Control (unless clinically indicated; group 1), metronomic CPM only (group 2), TroVax only (group 3), or CPM & TroVax (group 4). Randomization was undertaken at the Clinical Trials Office, University Hospital of Wales, using an un-stratified balanced block design, with the outcome communicated to the attending physician immediately upon randomization, but after participant enrollment. Treatment allocation was not masked in this open-label study.

Procedures

50mg CPM (Pharmacia Ltd.) was orally administered twice-a-day on treatment days-(TD)1-7 and 15-21 or until patient relapsed. Groups 2&4 patients were contacted by phone during CPM treatment to ensure compliance. $1x10^9$ $TCID_{50}$ TroVax (Oxford BioMedica) was administered as an intramuscular injection at TD22, 36, 50, 64, 78 and 106. Peripheral blood samples were taken at regular intervals (see schematic; figure 1).

We performed physical examinations and full blood count, urea and electrolytes, and liver function tests at each blood test visit. Tumor burden was assessed quantitatively using RECIST criteria following CT scans of the abdomen and chest at treatment week 12. Beyond 16-weeks of treatment, assessments were performed every 12-weeks until documented disease progression, whereby the patient would be treated with standard chemotherapy as indicated.

To assess immunological responses, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by centrifugation over Ficoll, and cultured in triplicate with 5T4 peptide pools or control antigens for 14-days. IFN-γ ELISpot assays were performed to assess for 5T4-specific T-cell responses, as previously described.[10] Positive responses were identified as having at least 20 spot-forming cells (SFC) per 10^5 cultured PBMC, and double the number of spots above background.

To perform T-cell counts, 3μl CD3-APC, CD4-PE and CD8-PerCPCy5.5 were added to 50μl of whole heparinized blood using a reverse pipetting technique. Red blood cells were lysed before addition of 50μl CountBright Beads (ThermoFisher); samples were acquired on a FACSCanto II (BD) and cell counts calculated according to manufacturer instructions.

To calculate the proportion of $CD4^+$ T-cells expressing Foxp3, fresh PBMC were stained with Live/Dead-Aqua, stained with CD4-APCh7, followed by fixation/permeabilization and intracellular staining with Foxp3-APC.

Plasma samples were collected from blood separated over Ficoll to measure 5T4-specific antibodies, determined using semi-quantitative ELISA, as previously described.[17] Polyclonal plasma positive for 5T4 was used as a standard curve for each assay. A 2-fold increase in 5T4 antibody relative units (RU) was established as the level at which a <1% false-positive rate could be expected, and antibody levels were considered positive above this value.

Outcomes

Given the trials' primary objective to measure the effect of TroVax and/or CPM on anti-tumor immune responses, the primary endpoint was the magnitude of 5T4-specific responses at TD43 (week 7). Secondary endpoints included the kinetics of anti-5T4 immune responses over time, PFS, OS, treatment-emergent adverse events, and Treg depletion during CPM treatment. PFS was defined as the time from date of trial randomization to the date of first documented tumor progression, as determined by evidence of radiological progression on CT scan, or clinical deterioration as assessed by the oncologist. OS was defined as the time from date of trial randomization to date of death due to any cause.

To assess safety, we reported adverse event occurrences to the Data Monitoring and Ethics Committee after every six completed patients in group 4. Safety in this group was assessed weekly for the first 4 weeks.

Statistical analysis

Follow-up is complete to Dec 13, 2016, whereby all patients had progressed or received other treatments. Power was based on the average effect size expected for each treatment, allowing for possible synergy between the two treatments. A randomization of 27 versus 27 patients to receive CPM or not gave 80% power to detect a moderate difference of 0.8 standard deviation in anti-tumor immune response, or other laboratory markers. For TroVax randomization, a total of 54 patients (allocated as 36 versus 18) gave 80% power to detect a difference of 0.83 points between TroVax or not. As the possible synergy between CPM and TroVax is of interest, the TroVax randomization is in a ratio of 2:1; if such synergy is seen, then a comparison of adding CPM to TroVax will contain 36 patients, enough to see a difference of 1 standard deviation with 80% power.

All analyses were performed on an intention-to-treat basis with patients ineligible for the trial excluded. Wilcoxon matched-pairs signed-rank tests were used to compare non-parametric datasets, including the assessment of Treg depletion during CPM treatment and 5T4 immune responses generated; patients were subdivided based on these results, according to endpoints stipulated in the trial protocol. OS and PFS were analyzed using log-rank tests and displayed using Kaplan–Meier plots. Categorical data analysis was performed using stratified Mantel-Haenszel tests. Effect sizes are displayed as Peto odds ratios with 95% confidence intervals. Analyses of each treatment were performed stratified for the other treatment allocation within the factorial design and stratified results displayed as Forest plots. A p-value less than 0.05 was considered statistically significant and all

tests of significance were 2-sided. Analyses were performed using SAS v9.4 and GraphPad Prism v7.

Results

Between July 2012 and February 2016, 55 patients were recruited and randomized. One patient from group 3 withdrew consent before receiving the allocated intervention, and one patient from group 1 and another from group 3 were later found to have undergone a curative procedure pre-enrolment (figure 2). These three patients were not included in the analyses of immune responses and PFS/OS, however an additional patient was recruited to group 3 and was included in the analyses, hence 52 patients could be evaluated. The baseline characteristics of the patients randomized to the four groups are shown (table 1). All patients presented with liver, lung and/or peritoneal metastases.

The key objective of the study was to determine the effect of low-dose CPM on anti-tumor immune responses, and whether such treatment could enhance immune responses generated by TroVax vaccination. CPM alone induced many immunological perturbations, most evident being a striking increase in IFN-γ^+ 5T4-specific T-cell responses, and the depletion of Foxp3$^+$ Tregs (24/27 group 2&4 patients (figure 3)); significant depletions in Tregs were noted during treatment week 3 at both TD15 (p=.01) and TD18 (p=.003), in comparison to TD1 (figure 3B). Setting a threshold of a decrease in absolute Treg numbers (i.e. %Treg depletion) above the upper 95%-CI interval (figure 3C), these patients exhibited prolonged median PFS over non-responding patients (HR=0.48 95%-CI 0.21-1.11; p=.09; figure 3D, and shown stratified by TroVax, eFigure 1). Hence effective CPM-induced Foxp3$^+$ Treg depletion was associated with prolonged PFS, but statistical significance was probably not reached due to sample size and the study not being powered to directly address this. Treg numbers return to baseline by TD29 in CPM-only treated patients and remained at this level for the duration of the trial (data not shown).

The presence of anti-5T4 immune responses was analysed throughout the trial. Significant increases in 5T4 antibodies were evident following two vaccinations at TD43 (group 3 vs.1,

p=.02, group 4vs.1, p=.002, figure 4A) hence the primary endpoint was met. Following this, 5T4 antibody levels increased further for many patients, e.g. treatment day 78 (group 3vs.1, p<.001, group 4vs.1, p=.003, figure 4A). Despite a trend for group 4 patients exhibiting larger increases over group 3 in anti-5T4 antibody titres (and corresponding anti-MVA titres; eFigure 2) at TD64, 78 and 106, this was not significant. Anti-MVA antibodies emerged at similar time-points, yet only small non-significant reductions were noted in 5T4 antibodies after TD64. When analysing secondary endpoints, development of 5T4 antibodies was consistent amongst TroVax-treated patients, with 15/17 group 3 and 13/18 group 4 patients mounting a >2-fold increase in anti-5T4 antibodies at some time during the trial (figure 4B). There was one instance of a CPM-only treated patient also mounting a doubled anti-5T4 antibody response during treatment (figure 4B).

The maximum increase in baseline 5T4-specific IFN-γ^+ T-cell responses revealed varying degrees of T-cell response to TroVax vaccination, with 20/35 patients mounting a >2-fold increase. Amongst groups 1&2, 10/17 patients mounted a >2-fold increase, owing mostly to CPM increasing anti-5T4 T-cell responses via Treg depletion (figure 4B).

When considering all trial participants that generated >2-fold increase in both anti-5T4 T-cell and antibody responses to CPM or TroVax at any instance during the trial (filled triangles/circles; figure 4B), this was associated with prolonged PFS (5.7 vs. 2.4 months, HR=0.58, 95%-CI 0.31-1.09, P=.09; eFigure 3A) and OS (20.0 vs. 13.1 months, HR=0.56, 95%-CI 0.28-1.12, p=.10; eFigure 3B). Amongst groups 3&4 TroVax-treated patients, this effect becomes more apparent since removing groups 1&2 patients from the analysis reveals a highly significant difference in PFS (5.6 vs. 2.4 months, HR 0.21, 95%-CI 0.090-0.47, p<.001; figure 4C) and OS (20.0 vs. 10.3 months, HR=0.32, 95%-CI 0.14-0.74, p=.008; figure 4D). Large increases in MVA titres to TroVax also significantly associated with PFS (HR=0.26, 95%-CI 0.11-0.59, p=.001; eFigure 2B&C) but not OS (p=.36; eFigure

2B&D), hence general immunological responsiveness of the patient may also determine outcome.

Although beneficial immunological responses to CPM or TroVax were independently associated with prolonged PFS over group 1, neither treatment was more effective than the other (eFigure1 and eFigure 4A&B), nor did combination group 4 patients exhibit improved survival over TroVax-only group 3 (figures 5A&B). 7/18 group 4 patients mounted 5T4 T-cell and antibody responses at any point in the trial, yet this was no better than in the 9/17 group 3 patients mounting similar responses to TroVax alone (p=.60; eFigure1A). Therefore, the addition of CPM does not enhance the effectiveness of TroVax, nor does it appear detrimental to boosting response to vaccination.

All three treatment groups demonstrated improved PFS but not OS over no treatment controls (figures 5C&D); overall survival data is difficult to interpret given that subsequent interventions offered to these patients is beyond our control. Group 2 patients demonstrated the greatest increase in median PFS over group 1 (HR=0.32, 95%-CI 0.094-1.09, P=.07; figure 5A), although the number of patients is low. Therefore, in mCRC patients, being treated with either TroVax or low-dose CPM was more effective than allowing a protracted chemotherapy “holiday”. In addition, there were no instances of treatment-related grade 3/4 adverse events (eTable 2), suggesting an excellent safety profile of these interventions.

Discussion

This randomized study demonstrated the clinical benefit of both a vaccine and low-dose CPM in the treatment of mCRC. Each treatment improved anti-tumor immunity via different mechanisms; CPM was effective at depleting Foxp3^{+} Tregs, resulting in boosted anti-5T4 T-cell responses, which associated with survival, and TroVax was effective at inducing cellular and humoral anti-5T4 responses, again to the benefit of patient PFS and OS in a subgroup analysis of responders versus non-responders. In combination however, CPM did little to improve anti-5T4 responses during TroVax treatment, despite a modest increase in anti-5T4 antibodies. This may reflect CPM blocking the priming and proliferation of other important immune cell subsets required for effective vaccination, e.g. tumor-antigen specific effector T-cells and dendritic cells[18,19] Further in-depth characterization is ongoing to decipher the exact effect of metronomic oral CPM on immunological responses, and how it might impinge on other cell populations.

Saito et al. recently hypothesised that depletion of Foxp3^{hi} suppression-competent Tregs abundant in CRC may provide clinical benefit,[20] and this trial appears to corroborate this. A number of studies have indicated a better prognosis when tumors are infiltrated with relatively high numbers of Foxp3^{+} Tregs.[21-23] Our previous studies demonstrated that as tumors advance, peripheral Treg proportion and suppressive capacity increase;[10] in addition Treg depletion significantly improves anti-tumor immune responses in mice[24] and CRC patients.[9,10,25] In this trial, when Tregs were most effectively depleted, median PFS doubled (2.5 to 5.0 months). Although our evidence is limited to depletion of peripherally-derived Foxp3^{+} Tregs, the prolongation in PFS indicates effective tumor control via the probable concurrent depletion of intratumoral Tregs, thus releasing the brake on intratumoral effectors. Hence it seems highly plausible that colorectal tumor-specific Tregs,

certainly in advanced disease, are detrimental to patients, and high pre-existing Foxp3$^+$ Treg infiltration is merely a bystander effect of a larger anti-tumor immune response.[26]

Regardless of mechanism, patients mounting anti-5T4 responses during CPM-treatment or TroVax vaccination were associated with a statistically significant increase in PFS and OS. In keeping with previous trials of TroVax, whereby intramuscular injection of $1x10^9$ $TCID_{50}$ TroVax induced the strongest immune response correlating with disease control,[17,27,28] the vaccine was shown to be safe, well-tolerated, and the induction of an anti-5T4 immune response did not result in any detrimental off-target autoimmunity.

Prior chemotherapeutic regimens in this metastatic CRC patient group appeared to have little effect on responses to either immunotherapeutic treatment (table 1), although some patients began the trial with relatively high pre-existing anti-5T4 responses, potentially induced by prior treatments. We found no evidence of pre-existing responses correlating with improved outcomes, although patients with higher pre-existing 5T4 antibody levels exhibited significantly higher 5T4 antibody responses to TroVax during treatment (data not shown).

Large increases in Tregs occurred in several patients regardless of prior CPM treatment, in particular amongst HLA-DR1$^+$/DQ5$^+$ patients following a single TroVax injection (data not shown). Whilst this factor, alongside increased MVA antibodies (eFigure2), initially suggested the induction of peripheral tolerance to TroVax, anti-5T4 T-cell and antibody responses remained largely unaffected. There was also no correlation between high anti-MVA titres and low anti-5T4 responses, although an association was identified between large increases in anti-MVA titres and PFS, but not OS (eFigure2), indicating that general patient health and immunocompetence may play a role in responsiveness to immunotherapy. Given that T-cell responses to the control antigen tuberculin-PPD varied little during the trial, nor associated with patient outcome (eFigure5), this suggests that the

key immune responses are those generated against tumor antigens, e.g. 5T4. This evidence supports exploring the use of TroVax (and other cancer vaccines[29]) earlier in the disease course.

In summary, this randomized trial identified a subset of 'immunotherapy-responsive' mCRC patients demonstrating better tumor control when given either CPM or TroVax. Although CPM failed to enhance TroVax immunogenicity, clear survival benefits with minimal side effects were demonstrated and further investigation is warranted. Given CPM's ineffectiveness in sustained Treg depletion during TroVax vaccination, we would propose the combination of TroVax with more potent blockade of tumor-derived immunosuppression for future development, for example with anti-CTLA-4 to eliminate intratumoral Treg,[30] or with anti-LAG-3 checkpoint inhibitors, given the extent of infiltration of highly suppressive LAG-3^{+}CD4^{+} tumor-infiltrating T-cells.[31]

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Author Contributions: Prof Godkin was the principal investigator and had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Scurr, Harrop, Hills, Gallimore and Godkin.

Acquisition, analysis, or interpretation of data: Scurr, Pembroke, Bloom, Roberts, Thomson, Smart, Bridgeman, Adams, Brewster, Jones, Gwynne, Blount, Harrop, Wright, Hills, Gallimore and Godkin. (Scurr, Pembroke, Roberts, Adams, Brewster, Jones, Gwynne and Godkin did the study procedures; Scurr, Bloom, Roberts, Thomson, Smart and Bridgeman performed IFN-γ ELISpot and flow cytometry; Blount and Harrop performed 5T4 ELISAs; Scurr, Pembroke, Bloom, Roberts, Thomson, Blount and Harrop collected data; Wright and Hills managed data; Scurr, Pembroke, Harrop, Wright, Hills, Gallimore and Godkin interpreted data.)

Drafting of the manuscript: Scurr, Hills and Godkin.

Critical revision of the manuscript for important intellectual content: Scurr, Pembroke, Roberts, Adams, Wright, Hills, Gallimore and Godkin.

Statistical analysis: Scurr, Wright, Hills and Godkin.

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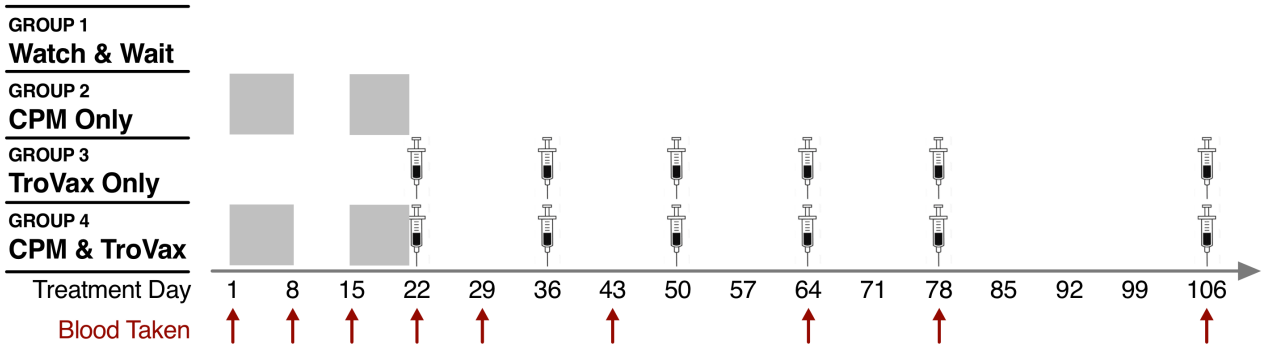

***Figure 1*. TaCTiCC treatment schedule.**

50mg CPM was given twice daily at treatment days 1-7 (week 1) and 15-21 (week 3) to groups 2 & 4. $1x10^9$ $TCID_{50}$ TroVax was given as an intramuscular injection at treatment days 22 (week 4), 36 (week 6), 50 (week 8), 64 (week 10), 78 (week 12) and 106 (week 16) to groups 3 & 4.

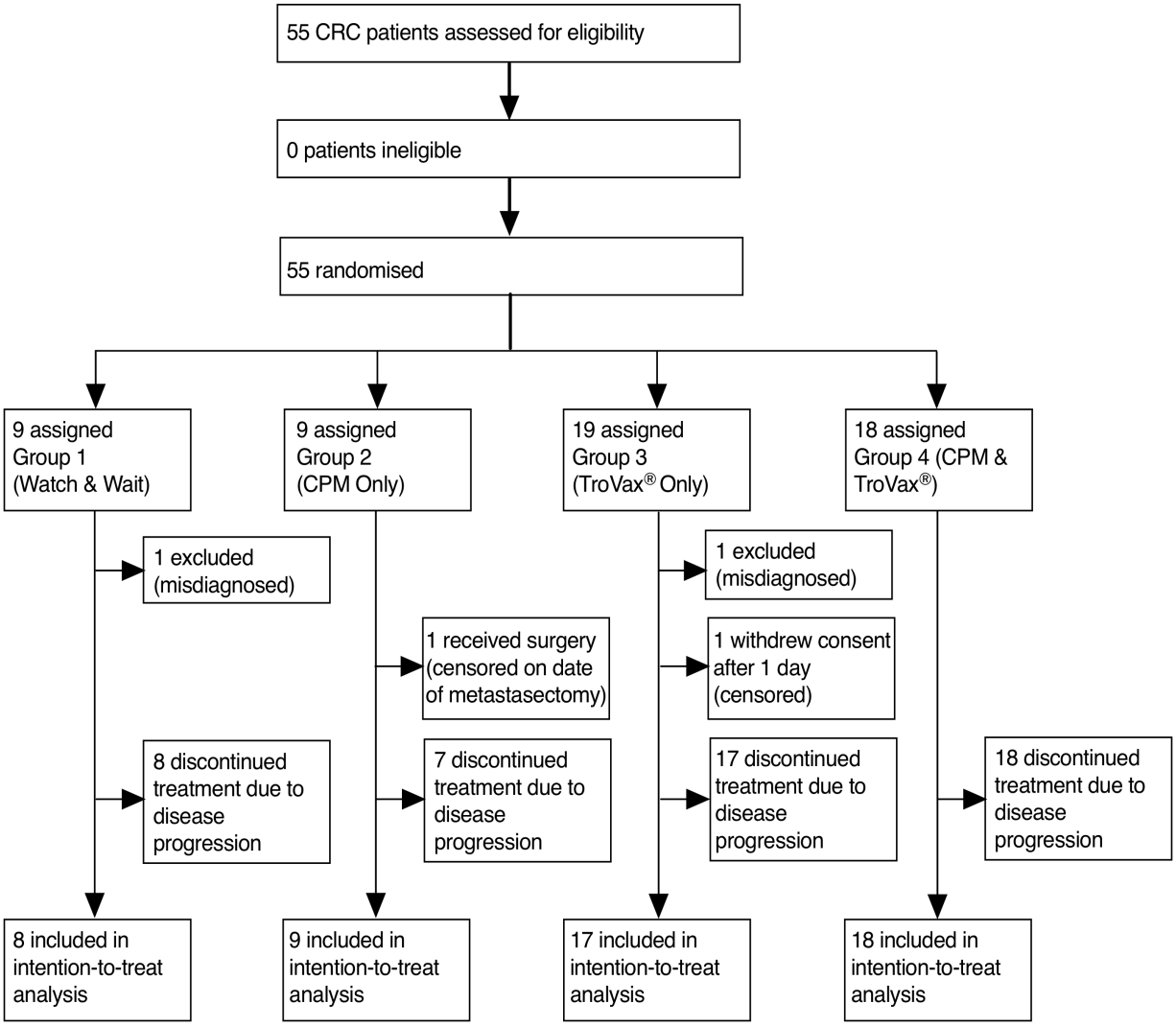

***Figure 2*. CONSORT flow diagram of study enrollment.**

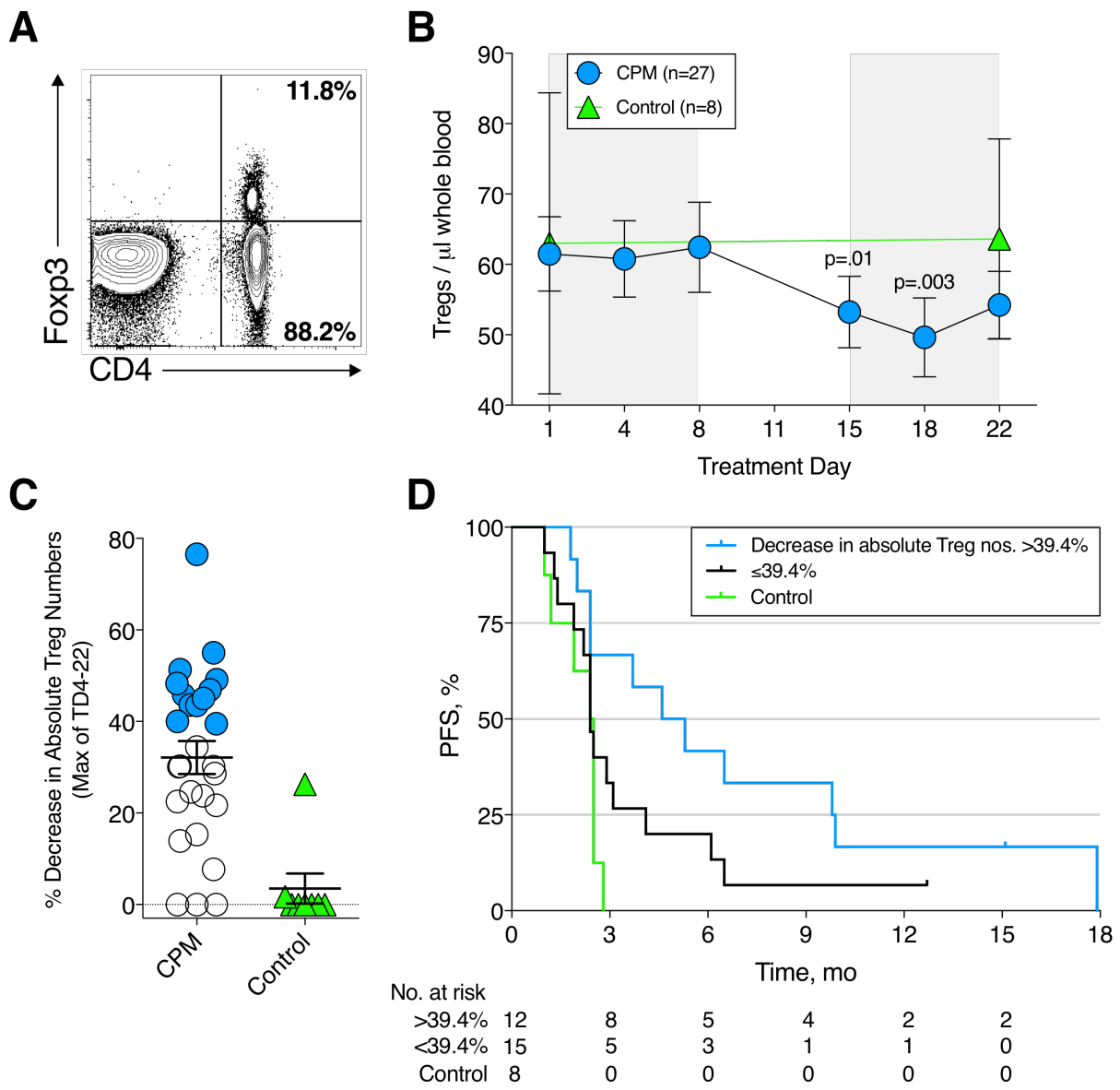

Figure 3. **Improved progression-free survival of patients with sufficient Treg depletions in response to CPM.**

Peripheral Foxp3$^+$ regulatory T-cells were enumerated by initially counting the CD3$^+$CD4$^+$ T-cells in whole blood, followed by phenotypic analysis to determine the proportion of CD4$^+$ T-cells expressing Foxp3, an example of the gating strategy is shown (A). Overall Treg numbers are shown at indicated time points during CPM treatment (B: Blue circles: CPM-treated patients (n=27); Green triangles: Control (group 1) patients (n=8)). The maximum % decrease in absolute CD4$^+$Foxp3$^+$ Treg numbers during TD4-22 when compared to baseline was measured (C). The threshold for positive response was set at

the upper 95%-CI interval of 39.4% Treg depletion, those patients meeting this criterion are highlighted in blue (n=12). (D) Kaplan Meier survival curves for three groups of patients: CPM responders (>39.4% Treg depletion; blue line, n=12); CPM non-responder (black line, n=15); control group (green line, n=8). The median PFS among CPM responders was 5.0 months, as compared to 2.5 months for poor / non-responders and 2.5 months for controls (HR 0.48 (95% CI 0.21-1.11); p=0.09).

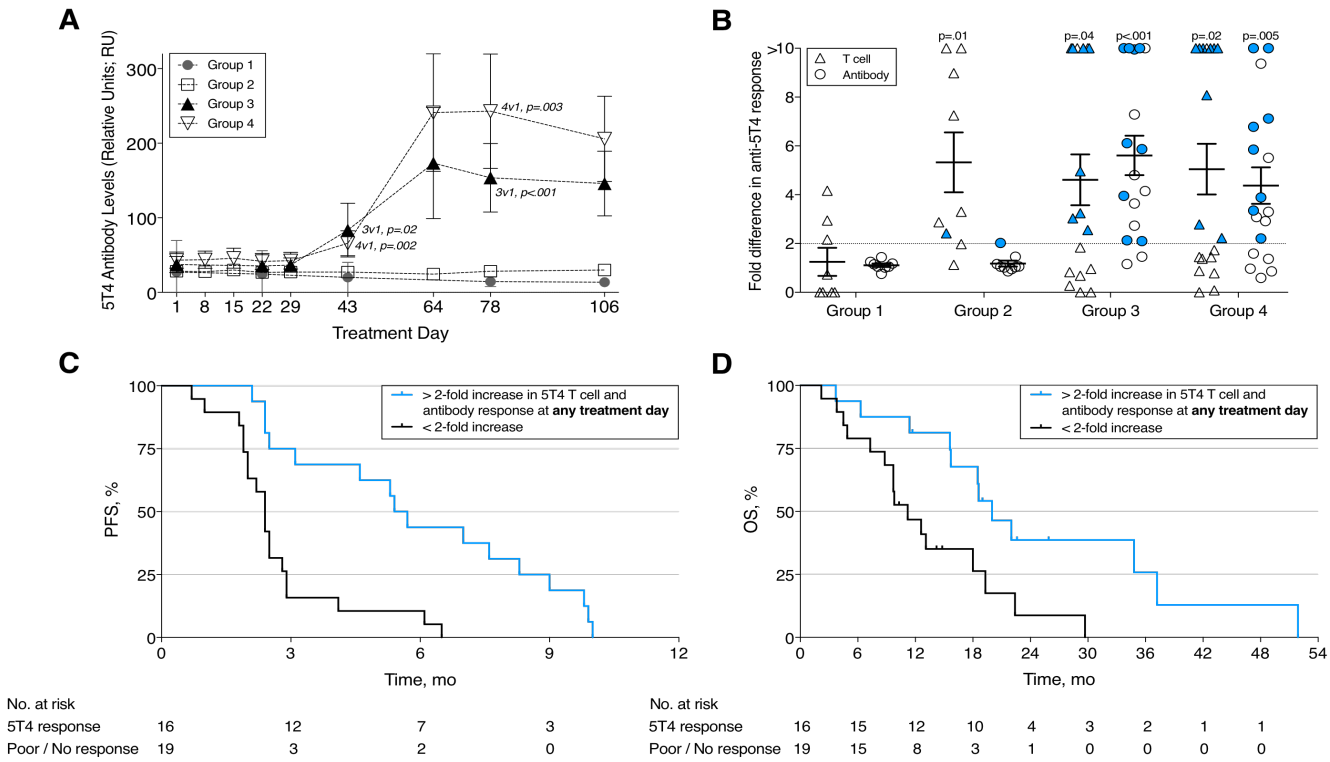

***Figure 4*. Anti-5T4 immunological responses associate with survival.**

(A) 5T4-specific antibody levels were measured from plasma samples taken throughout the course of the trial. (B) The fold increases in 5T4-specific IFN-γ^+ T-cell and antibody responses were calculated by dividing the highest response to treatment at TD8-106 by baseline (TD1) level. TroVax® recipients (groups 3 & 4) demonstrating a >2-fold increase in both anti-5T4 T-cell and antibody responses at any point during the trial are highlighted in blue (n=16). These patients exhibited significantly prolonged PFS (C: HR 0.21 (95% CI 0.090-0.47); p<0.001) and OS (D: HR 0.32 (95% CI 0.14-0.74); p=0.008) over TroVax® non-responders.

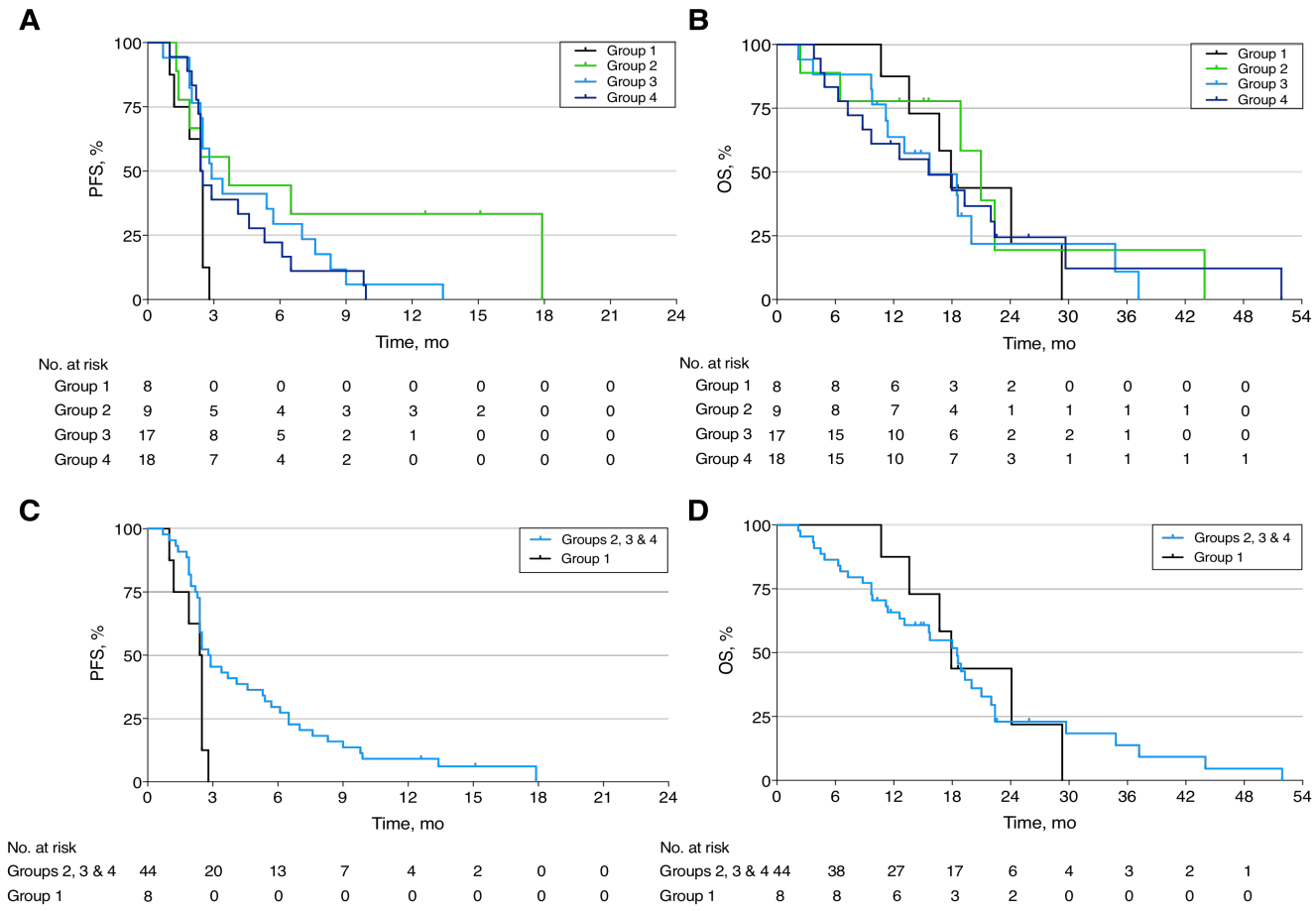

***Figure 5*. Survival by trial group.**

Survival outcomes of all patients by trial group is indicated for PFS (A) and OS (B). Survival outcomes of patients receiving any treatment (groups 2, 3 and 4, n=44) versus no treatment control (group 1, n=8) is indicated for PFS (C: HR 0.41 (95% CI 0.20-0.88); p=0.02) and OS (D: HR 1.03 (95% CI 0.43-2.47); p=0.95).