Rationally developing the next generation of personalised drug-chemoradiotherapy combination trials in anal cancer

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Biological features of Squamous cell carcinoma of the anus – a deep dive review. Authors: **Robert J Samuel,** Natalie Winder, Morten Busk, Karen Lycke Wind, Glen Guerra, Alexander G Heriot, Duncan C Gilbert, Andrew Macdonald, Karen-Lise Garm Spindler. This invited review was submitted on 11th March 2024 to ESMO Gastrointestinal Oncology. As of thesis submission in December 2024, other invited manuscripts have delayed publication of this special edition. Sections taken from this manuscript within the introduction are directly relevant to this thesis. I was involved in the conceptualisation, investigation, project administration, visualisation, writing – original draft, and writing – review & editing for all sections in the manuscript. Co-authors and I made figures used in the introduction. They are attributed appropriately.

The use of master protocols for efficient trial design to evaluate radiotherapy interventions: a systematic review. Authors: Alexandra Gilbert, **Robert Samuel**, Daniel Cagney, David Sebag-Montefiore, Julia Brown, Sarah R Brown, JNCI: Journal of the National Cancer Institute, April 2024, https://doi.org/10.1093/jnci/djae084. Sections directly relevant to this thesis have been taken from this manuscript within the introduction and have informed some of the conclusions in Chapter 5. I was involved in data curation, Formal analysis, writing—original draft and writing—-review & editing.

International consensus to define outcomes for trials of chemoradiotherapy for anal cancer (CORMAC-2): Defining the outcomes from the CORMAC core outcome set. Authors: **Robert Samuel**, Stephen R Knight, Richard Adams, Prajnan Das, Jennifer Dorth, David Finch, Marianne G. Guren, Maria A Hawkins, Susan Moug, Lakshmi Rajdev, David Sebag-Montefiore, Andrew G Renehan, Rebecca Fish plus the CORMAC-2 collaborators, eClinical Medicine December 2024, https://doi.org/10.1016/j.eclinm.2024.102939. Within the introduction, various sections directly relevant to the thesis have been taken from this manuscript. This work forms part of chapter 5. I was involved in the conceptualisation, data curation, formal analysis, investigation, project administration, writing of the original draft, and writing for the review and editing of all sections included in the manuscript. Together with Rebecca Fish and Andrew Renehan, I created the tables and figures taken from this manuscript.

Improving Outcomes with Chemoradiotherapy in the Mucosal Squamous Cell Carcinomas – Immune Checkpoint Inhibition and Broken Promises Authors: **Robert Samuel**, Adel Samson, Duncan C Gilbert. Clin Oncol (R Coll Radiol). 2023

Dec;35(12):764-768. doi:10.1016/j.clon.2023.09.003. Epub 2023 Sep 17. PMID: 37743210. Sections taken from this manuscript within the introduction are directly relevant to this thesis. I was involved in the conceptualisation, data curation, formal analysis, investigation, methodology, project administration, visualisation, writing – original draft, and writing – review & editing for all sections of the manuscript. I created all the tables and figures taken from this manuscript.

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Preface

The research environment in Leeds and Manchester and my background are relevant for this thesis. At the time of submission, I am a St4 Clinical Oncology Registrar. Since graduating from medical school in 2015, I have been involved in the NIHR integrated Clinical Academic Pathway. I began as an Academic Foundation trainee in Newcastle and then pursued an Academic Clinical Fellowship in Clinical Oncology in Leeds. The funding for this thesis comes from a CRUK Leeds-Manchester Clinical Research Training Fellowship. While I have always aspired to have a research career in cancer clinical trials, I had limited wet laboratory experience before this fellowship.

Leeds can claim to be a world leading centre in anal cancer. The PLATO trial is being run by the Leeds Clinical Trials Research Unit in Leeds. Professor Sebag-Montefiore is the Chief Investigator of PLATO, joint lead for ACT5 and lead author of the Research excellent framework 2021 impact case study on anal cancer. The University of Leeds and Manchester Cancer Research Centre have a long history of collaboration in anal cancer - much of the translational analysis for PLATO is being performed in Manchester; Professor Renehan is co-leading for ACT4 in PLATO, and Manchester led the CORMAC project with support from Leeds.

Until recently, there was very little research into the biology of anal cancer. Although PLATO has completed recruitment, planning for PLATO2 has already commenced. There have been calls from CRUK and other funders for future clinical trials to be at least biologically informed, if not biologically driven. The Samson group hold expertise in cancer immunology and translational immunotherapy. Until now, this expertise has not been applied to anal cancer.

Considering this background, this thesis aims to leverage expertise from Leeds and Manchester to enhance our understanding of anal cancer biology and to inform and drive the design of the next generation of anal cancer clinical trials. This aligns with my ambition to pursue a career in academic lower GI oncology and help bridge the gap between laboratory and clinical research, improving clinical care for patients with lower GI cancers.

Abstract

Anal squamous cell carcinoma (ASCC) is a rare HPV-associated cancer, with global trends indicating annual increases in incidence and mortality. Chemoradiotherapy (CRT) is the primary treatment for localised disease, yet 25-30% of patients with locally advanced disease experience relapse, highlighting the necessity for innovative therapies.

Given the critical role of the immune system in the response to CRT for ASCC and emerging evidence from metastatic disease, combining CRT with immunotherapy (IO) holds promise. Successful radiotherapy-immunotherapy (RT-IO) trials, such as PACIFIC and Checkmate 577 for lung and oesophageal cancer, respectively, have limited applicability to ASCC due to differences in biology, RT dose and elective nodal irradiation. Conflicting results from trials in other HPV-related cancers underline the need to investigate immune resistance markers in ASCC specifically rather than extrapolating from other cancers.

This thesis identified immune resistance markers associated with CRT in ASCC. Using flow cytometry to analyse peripheral blood, higher levels of peripheral regulatory T cells (Tregs) and increased expression of TIGIT and CTLA-4 expression were found to be associated with treatment failure six months after CRT. Cytokine analysis of plasma revealed no significant differences between treatment outcomes, indicating limited utility for future trials.

A systematic review identified eleven trials investigating RT-IO combinations in ASCC. All possible combinations of neoadjuvant, concurrent, and adjuvant IO were explored, utilising multiple types of IO. This review found substantial heterogeneity in current ASCC RT-IO trial designs, complicating future comparisons. Recommendations were made to reduce this heterogeneity in future trials. Heterogeneity in endpoint selection was addressed in CORMAC-2, which developed the first internationally agreed definitions for outcomes relating to disease activity and survival in CRT trials for ASCC treatment.

Targeting Tregs, CTLA-4, and TIGIT may enhance CRT efficacy, particularly with neoadjuvant and concurrent IO approaches. Future research should prioritise rigorous translational analyses and standardised methodologies to optimise IO integration in ASCC treatment.

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Abbreviations

3D-CRT	**3D conformal radiotherapy**
5-FU	**5-fluorouracil**
APOBEC	**Apolipoprotein B mRNA Editing Catalytic Polypeptide-like**
APR	**abdominoperineal resection**
ASCC	**Anal squamous cell carcinoma**
ATM	**Ataxia telangiectasia-mutated**
ATR	**Rad3-related protein**
CAR	**Chimeric antigen receptors**
CC	**Cervical cancer**
cGAS	**cyclic GMP-AMP synthase**
cHPV-DNA	**circulating HPV-DNA**
CisP	**Cisplatin**
COMET	**Core Outcome Measures in Effectiveness Trials**
CORMAC	**Core Outcome Research Measures in Anal Cancer**
COS	**Core outcome set**
CPS	**Combined positive score**
CRT	**Chemoradiotherapy**
CSCs	**Cancer stem cells**
CTCs	**Circulating tumour cells**
ctDNA	**circulating tumour DNA**
CTLA-4	**Cytotoxic T-lymphocyte associated protein 4**
Cutaneous T-cell Attracting Chemokine	**CTACK**
DAMPs	**Damage-associated molecular patterns**
DCs	**Dendritic cells**
DFS	**Disease-free survival**
d-MMR	**deficient mismatch repair**
EFS	**Event-free survival**
EGFR	**Epithelial growth factor receptor**
ENI	**Elective nodal irradiation**
Eosinophil Chemotactic Protein	**Eotaxin**
FFPE	**Formalin-Fixed Paraffin-Embedded**
Fibroblast Growth Factor, Basic	**FGF basic**
FRACTION	**Fast Real-time Assessment of Combination Therapies in Immuno-Oncology**
GM-CSF	**Granulocyte-macrophage colony-stimulating factor**

Granulocyte Colony-Stimulating Factor	**G-CSF**
Granulocyte-Macrophage Colony-Stimulating Factor	**GM-CSF**
GRECIAN	**identifyinG Radiotherapy Immune rEsistance meChanisms In Anal cancer**
Growth-Regulated Oncogene α	**GRO-α**
Hepatocyte Growth Factor	**HGF**
HNSCC	**Head and Neck squamous cell carcinoma**
HPV	**Human papillomavirus**
HRA	**High resolution anoscopy**
HR-HPV	**High-risk HPV**
HSIL	**High-grade squamous intraepithelial lesion**
H-TMB	**High tumour mutational burden**
ICD	**Immunogenic cell death**
ICIs	**Immune checkpoint inhibitors**
IFNs	**Interferons**
IFN-γ	**Interferon-γ**
IL	**Interleukin**
IMRT	**Intensity-modulated radiation therapy**
Interferon Gamma-Inducible Protein 10	**IP-10**
Interferon-α2	**IFN-α2**
Interferon-γ	**IFN-γ**
Interleukin 1 Receptor Antagonist	**IL-1ra**
Interleukin 10	**IL-10**
Interleukin 12 (70 kDa)	**IL-12 (p70)**
Interleukin 12 Subunit Alpha (40 kDa)	**IL-12 (p40)**
Interleukin 13	**IL-13**
Interleukin 15	**IL-15**
Interleukin 16	**IL-16**
Interleukin 17A	**IL-17A**
Interleukin 18	**IL-18**
Interleukin 2	**IL-2**
Interleukin 3	**IL-3**
Interleukin 4	**IL-4**
Interleukin 5	**IL-5**
Interleukin 6	**IL-6**
Interleukin 7	**IL-7**
Interleukin 8	**IL-8**
Interleukin 9	**IL-9**
Interleukin-1α	**IL-1α**

Interleukin-1β	**IL-1β**
Interleukin-2 Receptor α	**IL-2Rα**
IO	**Immunotherapy**
Leukaemia Inhibitory Factor	**LIF**
LRF	**Loco-regional failure**
Macrophage Colony-Stimulating Factor	**M-CSF**
Macrophage Inflammatory Protein 1 β	**MIP-1β**
Macrophage Inflammatory Protein-1α	**MIP-1α**
Macrophage Migration Inhibitory Factor	**MIF**
MAMS	**Multi-arm, multi-stage**
MHC	**Major histocompatibility complex**
MMC	**Mitomycin-C**
Monocyte Chemoattractant Protein-1	**MCP-1**
Monocyte Chemoattractant Protein-3	**MCP-3**
Monokine Induced by γ Interferon	**MIG**
MRD	**Microscopic residual disease**
MSI	**Microsatellite instability**
MSM	**Men who have sex with men**
NK cells	**Natural Killer cells**
NLR	**Neutrophil-lymphocyte ratio**
NSCLC	**Non-small cell lung cancer**
OARs	**Organs at risk**
ORR	**Objective response rate**
OS	**Overall survival**
p16	**p16INK4a**
PD-1	**Programmed cell death protein 1**
PD-L1	**Programmed death ligand 1**
PDOs	**Patient-derived organoids**
PDX	**Patient-derived xenografts**
PFS	**Progression-free survival**
Platelet-Derived Growth Factor-BB	**PDGF-BB**
PLATO	**PersonaLising Anal cancer radioTherapy dOse**
PLHIV	**Persons living with HIV**
pRB	**Retinoblastoma protein**

Regulated upon Activation, Normal T-cell Expressed and Secreted	**RANTES**
ROS	**Reactive oxygen species**
RT	**Radiotherapy**
RT-IO	**Radiotherapy-immunotherapy**
RTQA	**Radiotherapy quality assurance**
SABR	**Stereotactic ablative radiotherapy**
SCCs	**Squamous cell carcinomas**
SMART	**Stereotactic Magnetic Resonance Guided Adaptive Radiation Therapy**
Stem Cell Factor	**SCF**
Stem Cell Growth Factor β	**SCGF-β**
STING	**Stimulator of interferon genes**
Stromal Cell-Derived Factor 1 Alpha	**SDF-1α**
TAAs	**Tumour-associated antigens**
TCR	**T-cell receptor**
TGF-β	**Tumour growth factor-β**
TIGIT	**T-cell immunoreceptor with immunoglobulin and ITIM domain**
TILs	**Tumour infiltrating lymphocytes**
TME	**Tumour microenvironment**
TNF-Related Apoptosis-Inducing Ligand	**TRAIL**
TPS	**Tumour proportion score**
Tumour Necrosis Factor α	**TNF-α**
Tumour Necrosis Factor β	**TNF-β**
Vascular Endothelial Growth Factor	**VEGF**
VMAT	**Volumetric arc therapy**
β-Nerve Growth Factor	**β-NGF**

Chapter 1 Introduction

1.1 Anal Cancer

1.1.1 Epidemiology

There were an estimated 54000 cases of anal squamous cell carcinoma (ASCC) in 2022, with global trends showing annual increases in incidence and mortality[1,2]. In the UK, incidence has increased by 76% since the early 1990s and doubled in the USA between the late 1970s and early 2010s[3,4]. Regardless, ASCC remains rare, with about 1,500 new cases each year in the UK, accounting for less than 1% of all new cancer cases[5].

Like cervical cancer (CC), ASCC is preceded by a high-grade squamous intraepithelial lesion (HSIL), and this can be detected by screening using high-resolution anoscopy (HRA). Anal HSIL encompasses the conditions previously termed anal intraepithelial neoplasia grades 2 and 3. Increased screening in high-risk groups such as those with HIV, men who have sex with men (MSM), immunocompromised populations and women who have had previous cervical, vaginal or vulval cancer may account for the increased incidence of early-stage disease. However, these screening programmes are inconsistent in geography and what constitutes a high-risk population. There is also a similar increase in later-stage disease across the same period, suggesting screening is not the only reason for this increasing incidence[6]. Appropriate screening programmes and their potential benefit is an active research area in ASCC but is outside the scope of this thesis.

1.1.2 Anal cancer risk factors

For most of the population, increasing age and female sex are the most important risk factors for ASCC, with a peak incidence of 80-84 years in the UK and 1.5 female cases to every male case in Europe[3,5]. Particular populations have a much higher incidence. Persons living with HIV (PLHIV), MSM, females with a history of gynaecological (pre)cancers, and non-HIV immunosuppressed individuals all have incidence per person-years higher than that of females without other risk factors[7]. Figure 1.1, taken from Stier et al. 2021[7], shows the relative incidence of ASCC in different populations, demonstrating the combined impact of HIV status and MSM on incidence as well as the effect of age on other risk factors. Given the small size of these populations in the UK and other developed countries compared to the population without these risk factors, most patients with ASCC are not MSM and are not PLHIV. Recent Human papillomavirus (HPV) infection and smoking are other important risk factors[8]. Smoking is not only associated with an increased incidence of ASCC but with a worse outcome following chemoradiotherapy (CRT) treatment[9].

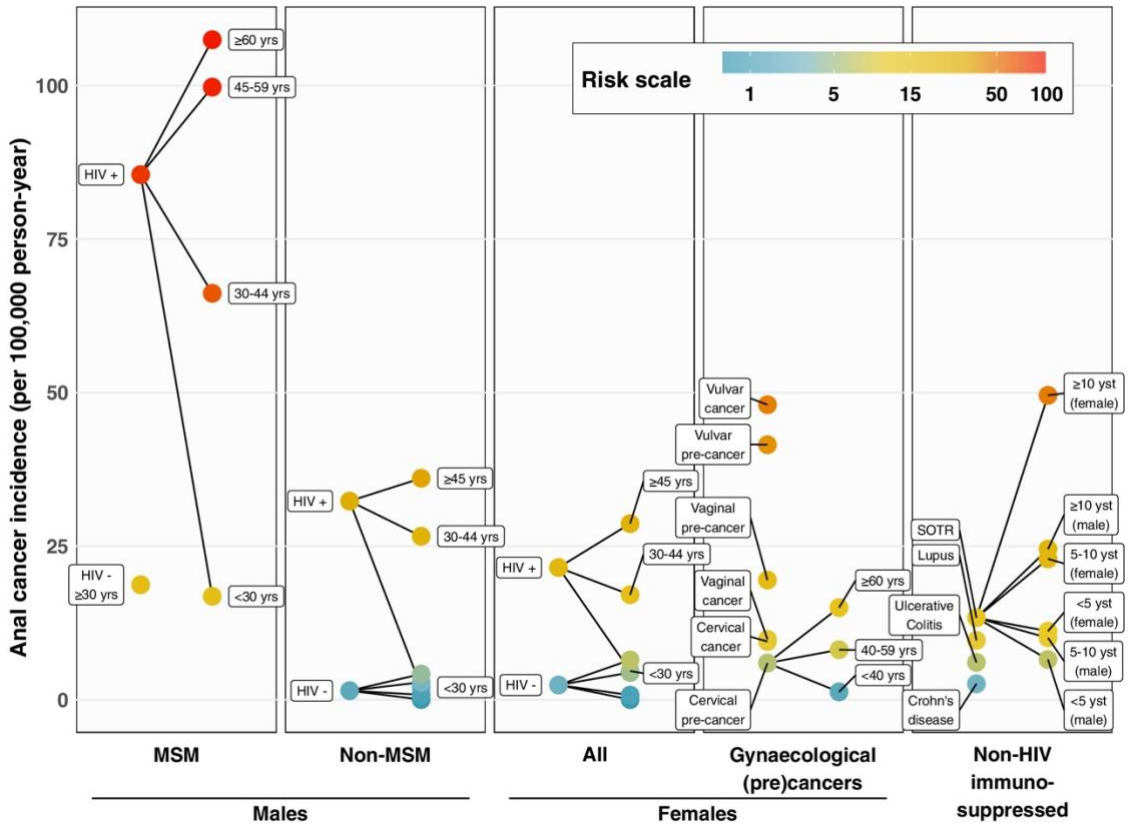


Figure 1.1 Relative incidence of ASCC for different populations. Taken from Stier et al 2021[7].

1.1.3 Prognostic Factors for Anal Cancer

In a systematic review of 19 studies reporting prognostic factors, T stage, N stage and sex were the most prevalent and reliable clinical parameters for survival and cancer-related outcomes after CRT for ASCC[10]. Leucocytosis, neutrophilia, and anaemia at baseline were all haematological biomarkers identified as prognostic in more than one study. Although the evidence base is relatively small, it is common for anaemic ASCC patients to receive blood transfusions before commencing CRT. HPV status is the strongest prognostic biomarker, with HPV+ disease ASCC patients having a better prognosis than HPV- patients. Higher HPV viral load in the baseline tumour is also associated with better prognosis[11].

1.1.4 Role of HPV in Anal Cancer

HPV is a small (50-80nm), non-enveloped virus with a circular double-stranded DNA genome 8 kb in length (Figure 1.2)[12]. HPV infections predominantly occur through skin-skin or skin-mucosa interaction, with sexual transmission being the most documented cause. HPV enters the host directly through the epidermis and mucous membranes. Approximately 85% of individuals who are HPV+ will be asymptomatic on initial infection, with 15% experiencing symptoms such as skin lesions/warts. Reoccurring or persistent infection, especially with high-risk HPV (HR-HPV) types, can lead to cellular

dysplasia and eventually cancer. The proportion of HPV-attributable cancers at each anatomical site varies, from 99.7% for CC to about 2% for oral cavity cancers[13]. At least 90% of ASCCs are HPV-associated, with p16INK4a (p16) immunohistochemistry used to infer HPV causation clinically. High-sensitivity genotyping suggests this figure may be higher and closer to the frequency seen in CC[14]. HPV16 is the most common HR-HPV genotype, accounting for about 55% of ASCC, followed by HPV18 (15%), with HPV31, 33, 45, 52, and 58 accounting for a combined additional 17%[13]. The HPV E6 and E7 genes encode open-reading frame proteins that form complexes with host cell proteins, leading to the inactivation of tumour suppressor p53 and retinoblastoma protein (RB), respectively. They are the only viral genes always retained and expressed in HPV+ cancers with cellular senescence induced in experiments when inhibited[15–18]. Figure 1.3 show these pathways in detail.

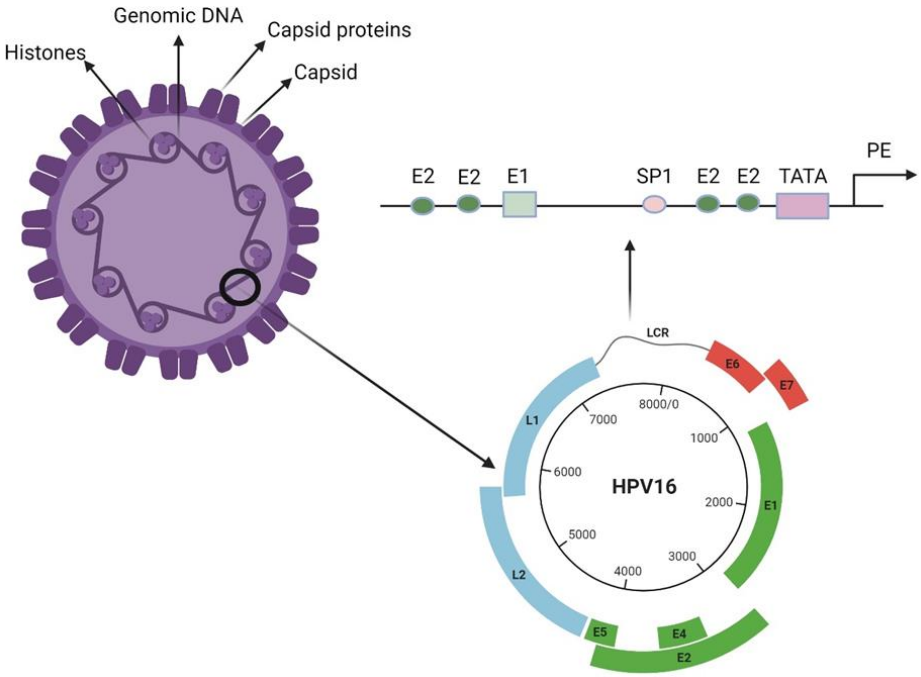


Figure 1.2 Structural architecture of HPV, alongside the organization of the HPV genome and sequence of viral gene expression. Made by Natalie Winder.

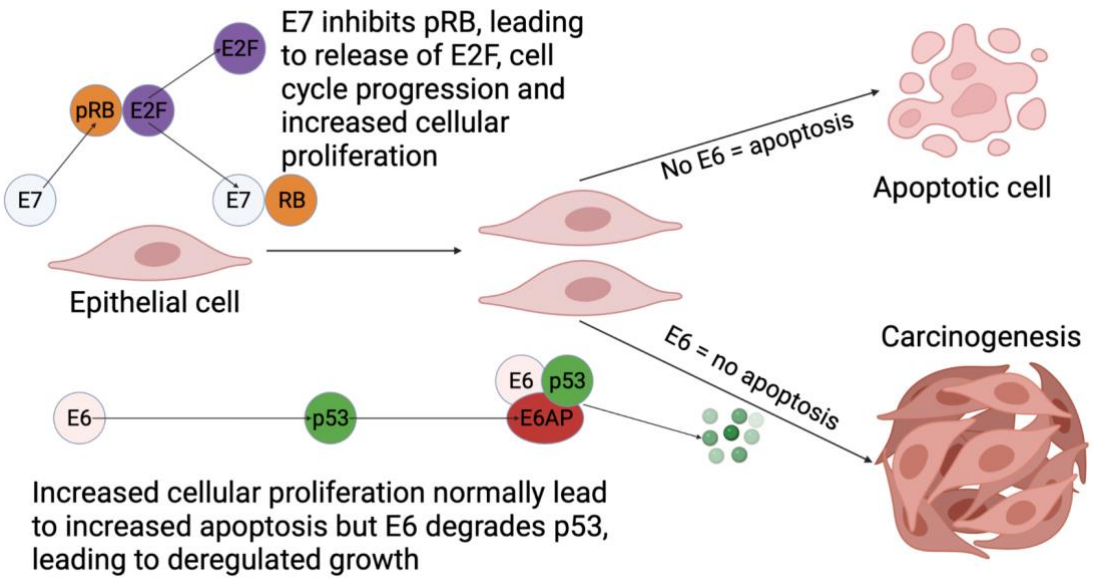


Figure 1.3 E6 and E7 oncogenesis. E6 binds with E6-associated protein (E-6AP) and then to p53, causing degradation of p53. E7 binds to pRB, functionally inactivating it, releasing E2F and promoting the cell cycle.

1.1.5 Impact of HPV vaccination on incidence

The HPV vaccination has emerged as one of the most significant public health achievements of the 21st century. Based on research conducted by Zhou and Frazer in Queensland during the 1990s, the original Gardasil vaccine was designed to protect against HPV strains 6, 11, 16, and 18, and received FDA approval in 2006. In the UK, an HPV immunisation program was launched in 2008, initially targeting girls aged 12 to 13. The program provided a catch-up opportunity for older girls, up to 18 years old in 2009, and it was expanded to include boys aged 12 to 13 in 2018.

The programme is already having an impact on the incidence of CC and high grade cervical intraepithelial neoplasia, with an estimated 687 CC cancer cases (95% CI 556 to 819) and 23,192 (22,163 to 24,220) cases of CIN3s prevent by the mid-2020s, reducing risk by 87%. The early impact of CC is linked to its younger age of incidence compared to other HPV-associated cancer, and a reduction in ASCC is not expected for 20-30 years. The COVID-19 pandemic led to a decline in HPV vaccination rates and to achieve the greatest reduction in ASCC, further research is needed to understand the reasons behind vaccine hesistancy[19,20].

1.1.6 The importance of the immune system

A higher incidence in people with HIV and solid organ transplant recipients empirically shows the importance of the immune environment for ASCC[7]. Elevated neutrophil-lymphocyte ratio, neutropenia, and leucocytosis have all been found to be negatively prognostic for ASCC and proposed as potential biomarkers for ASCC, highlighting the importance of the host immune response even without significant pharmacological or

pathological immunosuppression[21,22]. Furthermore, since it can take up to 6 months after finishing CRT to reach a complete response in ASCC, a degree of immune clearance is likely needed to achieve a complete response[23]. Failure of immune clearance may explain some loco-regional failures (LRFs) in the first few months after CRT, indicating that there could be a window of opportunity to intervene and improve outcomes. Viral oncoproteins E6 and E7 are tumour-specific antigens against which tumour infiltrating lymphocytes (TILs) are frequently specific in HPV+ cancers. CD8+ TILs from CC and Head and Neck squamous cell carcinoma (HNSCC) cells can be stimulated using HPV antigens, but this analysis has not been performed in ASCC[24,25]. In ASCC, the prognostic value of TILs was demonstrated in 2 combined cohorts of patients from the UK and Denmark, with TILs classified into high, medium, or low infiltration[26]. It showed that the TILs score added to the p16 status for the prognostication of ASCC patients receiving CRT. The complexity of the combined feature of HPV status and TILs highlights the need for a deeper understanding of the immune environment in ASCC.

Downregulation of MHC molecules, a skew towards a Th2 CD4+ response, and inhibition of cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) have all been proposed as immune evasion mechanisms in HPV+ CC and HNSCC. Still, there is currently no ASCC-specific data on HPV-associated immune evasion mechanisms.[27] The PD-1/PD-L1 axis is a mechanism of immune evasion in some cancers and is used as a biomarker for response to immunotherapy (IO)[28]. Depending on the study, 50-65% of ASCCs express programmed death ligand 1 (PD-L1), and most studies show that high PD-L1 correlates with improved survival after standard CRT. However, scoring systems and staining differences make results challenging to interpret and compare[29–31]. In localised ASCC, there is conflicting data regarding the association of outcomes with higher HPV viral load and high programmed cell death protein 1 (PD-1) expression[11,32–34].

Microsatellite instability (MSI), deficient mismatch repair (d-MMR), and high tumour mutational burden (>10 mutations/megabase (H-TMB)) are associated biological features that suggest high genomic instability. These are used as markers for response to IO in other cancers. For ASCC, the translational analysis from the CARACAS study showed that out of 40 available cases, 5 had H-TMB, one had MMR, and 10 of the available 52 cases expressed PD-L1+ (combined positive score (CPS) >40).[35] Given the low frequency of mutations in these specific genes involved in DNA damage repair, they do not appear to drive the immune response in ASCC. Instead, the integration of HPV into the host genome, resulting in the expression of viral antigens, appears to drive the host immune response in ASCC.

1.1.7 Biology and radiobiological features of HPV-positive and negative disease

Regardless of HPV status, the ability of squamous cells to rapidly repopulate after injury allows for accelerated repopulation after radiotherapy (RT) and is an essential feature

in radioresistance. This is supported by data suggesting that unscheduled treatment interruptions that increase overall treatment time negatively impact outcomes[36]. In contrast to other HPV-associated squamous cell carcinomas (SCCs), hypo- and hyper-fractionated RT regimens have not been investigated for ASCC.

Across HPV-associated cancers, HPV- cancer has a worse prognosis than HPV+ cancer, and this may be partly due to differences in response to RT. Much of the data on the differences in biology between HPV+ and HPV- disease has been derived from CC and HNSCC. Upon integration, HPV alters DNA repair pathways such as ataxia telangiectasia-mutated (ATM) and Rad3-related protein (ATR). Although HPV requires a low level of ongoing DNA repair for survival, changes in these pathways lead to the buildup of point mutations and insertions, along with numerical and structural changes in chromosomes. This facilitates ongoing G2/M cycle arrest, a phase in the cell cycle especially vulnerable to RT [37,38]. Data from CC shows that DNA damage generated from direct or indirect ionisation enables permanent damage within the cells due to their compromised ability to repair dsDNA breaks, leading to increased radiosensitivity.[39] In HNSCC, the survival fraction after 2 Gy (SF2) is 0.22 for HPV+ cell lines vs 0.59 for HPV– cell lines[40]. In addition, the HPV+ tumour microenvironment (TME) is characterised as more pro-inflammatory after RT, which may enhance its anti-tumour effects[41]. Impaired DNA repair mechanisms resulting from HPV manipulation of signalling pathways can also influence radiosensitivity by altering the metabolic activity within tumours, which can reduce tumour hypoxia and resident cancer stem cells (CSCs). HPV- HNSCC cancers show a 49% increase in resident CSCs compared to HPV+ cancer, resulting in a reduced cellular immune response[42]. CSCs are usually located within hypoxic areas of tumours and have been shown to enhance the repair of DNA damage more effectively in HPV- tumours than HPV+ tumours, which contain more differentiated tumour cells[43]. RT efficacy heavily relies on oxygenated areas to enable the formation of oxygen-free radicals. HPV's host cell transformation can enhance DNA damage from RT through viral oncoproteins E6 and E7. E6 can reduce the number of antioxidants within the TME, while E7 inhibits glutathione transferase, a protein whose function is to scavenge free radicals, enabling the accumulation of reactive oxygen species (ROS) (Figure 1.4)[12,44].

HPV- disease can occur through spontaneous mutations, exposure to certain stimuli (e.g., smoking) and hereditary conditions[45,46]. In HPV- HNSCC, carcinogenic nitrosamines from tobacco covalently bind to DNA, forming DNA adducts that produce double-strand DNA breaks, leading to hypermutations and chromosomal instability. This, in turn, dysregulates homeostatic pathways within the cells, such as the Fibroblast growth factor receptors and Epithelial growth factor receptor (EGFR) signalling, which subsequently affect RAS/RAF/ERK, PI3K and JAK/STAT pathways, resulting in uncontrolled cellular proliferation[47,48]. Smoking is a risk factor for HPV- HNSCC, and it can be extrapolated that similar pathways are responsible for HPV- ASCC.[49,50]

HPV+ and HPV- cancers are immunologically distinct, with HPV- CC, ASCC, and HNSCC having lower TILs[26,51,52]. Additionally, activation/exhaustion markers on B-cells, T-cells, and T-cell receptor (TCR) diversity are significantly lower in HPV- tumours[53]. Integration of HPV E6/E7 into the host genome leads to their de-regulated expression and further inactivation of the tumour suppressors p53 and pRB, respectively, preventing cell cycle arrest and reducing sensitivity to apoptotic signaling[48].

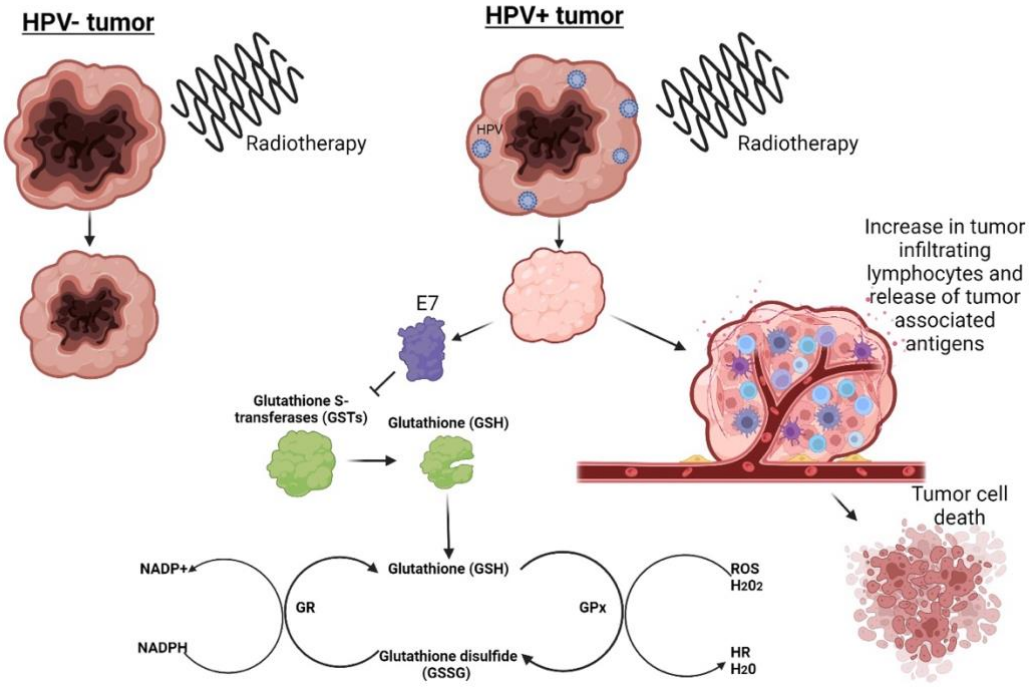


Figure 1.4 How HPV infection generates radiosensitive tumours. E7 viral oncoprotein can inhibit the function of detoxification enzymes, preventing the removal of free radicals and increasing the reactive oxygen species within the tumour, leading to tumour cell death. Made by Natalie Winder

1.1.8 Molecular characteristics

Mutations in *PIK3CA* and *FBXW7* are the most common in HPV+ disease, whereas *TP53* and *CDKN2a* are most common in HPV- disease. These findings are relatively consistent across different ethnic groups, stages of disease, pre-CRT versus post-CRT, and metastatic and localised disease[30,31,54]. *PIK3CA* mutations are often in exons 2 and 4 at hotspots known to generate oncogenic activity of *PIK3CA/mTOR* signalling[55]. Apolipoprotein B mRNA Editing Catalytic Polypeptide-like (APOBEC) is a family of enzymes involved in innate immune defence against viral infections, including HPV, causing hypermutations in viral DNA via deamination[56]. However, these enzymes can unintentionally cause host genome mutations, which may play a role in specific *PIK3CA* mutations in HPV+ ASCC. Although drugs targeting *PIK3CA* are approved for other cancers, there are currently no trials in ASCC.

EGFR overexpression is common in ASCC. Anti-EGFR therapies have shown efficacy in metastatic ASCC, with small benefits when combined with CRT [57,58]. However, side effects are substantial, and none of these trials were stratified for EGFR expression, limiting their clinical utility.

Despite recent improvements in molecular characterisation, there is currently little precision medicine for ASCC and no validated biomarkers to stratify treatment. Even HPV status, the most basic and well-characterized "molecular profile" of ASCC, is not used to tailor CRT, although it is currently being investigated in a Canadian trial[59].

Overall, there is an unmet need for ASCC-specific clinical trials to develop target-specific therapies for future patients, selecting or stratifying for biomarkers with extensive exploratory analysis to maximise learning from each patient. A recent review by Abba and colleagues comprehensively describes the advances in genomics, epigenomics, transcriptomics, and proteomics in more detail, providing insights for developing ASCC precision medicine[60]

1.1.9 History of anal cancer trials and current standard of care

Historically, radical surgery with colostomy was the only treatment option for ASCC. In the mid-1970s Nigro and colleagues treated three patients with 30Gy of RT, with 2 of them receiving concurrent mitomycin-C (MMC) and 5-fluorouracil (5-FU) chemotherapy. All three patients experienced tumour regression. A more extensive case series followed in the 1980s, again showing benefit[61]. However, it was not until the first generation of randomised phase III trials in the 1990s that CRT was established as the standard of care for ASCC. ACT1 and the EORTC trials investigated CRT versus RT alone, showing the superiority of CRT[62,63]. RTOG 87-04 found the addition of MMC to 5-FU CRT improved outcomes[64]. A second generation of Phase III trials followed in the 2000s-2010s. RTOG 98-11 investigated the addition of induction chemotherapy with 5-FU and cisplatin to CRT[65]. Using a 2x2 factorial design, ACCORD-03 investigated the same induction regimen and the addition of a local RT boost[66]. A 2x2 factorial design was also used in ACT2 to investigate concurrent cisplatin versus MMC and the addition of maintenance chemotherapy[67]. None of these trials showed evidence of a benefit from additional chemotherapy and did not change the standard of care.

These trials used higher doses of RT compared to the original Nigro case reports due to observed improved response rates but at the cost of acute and long-term toxicities. Many of these trials individually stratified for disease stage, identifying groups of patients with higher or lower risk of treatment failure based on TNM staging. Unlike current intensity-modulated radiation therapy (IMRT) practice, which involves continuous treatment, each trial included a 3–6-week gap between the initial RT and RT boost. Apart from ACCORD03, RT dose was not investigated within trials but was different across trials. In ACCORD03, an additional RT boost 3 weeks after completion of standard CRT to patients already responding to treatment. Heterogeneity in patient selection, stage of disease, RT technique, RT volumes, and primary outcomes have

made direct comparisons of RT dose for the same stage of disease challenging. This has led to different recommended CRT treatment regimens between countries for the same stage of disease. Current UK practice is 50.4Gy in 28 fractions to gross disease for $T_{1\text{-}2}N_0$ tumours and 53.2Gy in 28 fractions over 5.5 weeks for $T_{3\text{-}4}N_0$ or $T_{any}N_1$ disease, with planned weekend treatment breaks. Both are given with 12mg/m^2 of MMC given on day 1 with either 825mg/m^2 BD of capecitabine (only taken on RT days, not at weekends) or 1000mg/m^2 of 5-FU for 4 days at the start of weeks 1 and 5 of RT.

In contrast to these large Phase III trials, the use of Capecitabine as an option to replace 5-FU as part of standard of care was established from EXTRA - a small, multicentre randomised Phase II trial in the UK.[68] Doses of chemotherapy are reduced, or RT given alone depending on a patient's comorbidities and performance status. Agnostic to RT dose, improvements in RT techniques have reduced the dose to organs at risk, reducing the side effects of treatment. Initially, three to four 2-dimensional fields were used in the era of ACT1 and ACT2. This was replaced with 3D conformal RT (3D-CRT), which has been replaced by IMRT, used today in most modern Western cancer centres. Data from the UK indicates relatively mild symptomatic complications after one year of using IMRT compared to 3D-CRT[69].

Based on the above, concurrent CRT with 5FU/CAP and MMC without induction or maintenance chemotherapy was first trialled almost 50 years ago and has been the unchanged standard of care for at least 30 years[70].

1.1.10 PLATO trial

Building on the experience of ACT1 and ACT2 and the unanswered question of the optimal dose of RT for different stages of the disease, the CRUK-funded PersonaLising Anal cancer radioTherapy dOse(PLATO) trial is a national umbrella clinical trial led by the Leeds Cancer Research UK Clinical Trials Unit (CTU) at the University of Leeds. It comprises three trials stratified for risk of LRF: low-risk (ACT3), intermediate-risk (ACT4), and high-risk (ACT5) patients[71]. All three have finished recruitment and primary endpoint data is awaited.

ACT3 is a Phase II trial investigating patients with small tumours of the anal margin or canal that have been treated with local excision. Currently, there is no prospectively collected data on this patient cohort. If the excision margin is >1mm, the patient receives no further treatment, and if ≤1mm, they have 41.4Gy in 23 fractions.

ACT4 is a randomised phase II trial investigating standard-dose RT (50.4Gy) versus lower-dose RT (41.4Gy), both with standard chemotherapy, in patients with T1/2 disease without nodal involvement that have not had local excision prior to CRT.

The objective of both ACT3 and ACT4 is to determine whether dose de-escalation results in acceptably low LRF levels with reduced acute and late toxicity.

ACT5 is a seamless pilot, phase II and phase III trial comparing standard dose (53.2Gy) CRT with two experimental arms (58.8Gy and 61.6Gy) to determine whether dose escalation reduces three-year LRF in patients with T1/2 disease with nodal involvement or T3/4 disease.

Despite standard dose RT, around 30% of patients with high-risk disease will experience disease relapse after standard CRT, potentiated by tumour resistance mechanisms to RT[70]. Although ACT5 will determine whether RT dose escalation can reduce LRF, alternative treatment strategies are needed to address this resistance. These alternative treatment strategies are being investigated in this PhD.

1.1.11 Treatment morbidity

During and immediately after CRT, nausea, anal pain, skin erythema, diarrhoea and haematological toxicities are the most acute common adverse events[69]. In the 1990s, the 3-6 week gap during treatment was partly due to the severity of these acute side effects, with patients often requiring hospital admission during treatment. This planned treatment break was removed as RT techniques improved. 3D-CRT to IMRT and, latterly, volumetric arc therapy (VMAT) has sequentially reduced RT dose to pelvic organs at risk (OARs), reducing side effects and unplanned treatment breaks. Reductions in acute side effects are not only essential to reduce unplanned treatment breaks and reduce impacts on short-term quality of life but also to reduce the incidence of late side effects that can manifest months or years after CRT is finished. Sexual function, diarrhoea, urinary incontinence, and bowel incontinence due to RT fibrosis are the most common long-term toxicities associated with a substantial drop in quality of life[72]. Despite improvements since the 1990s, further work is needed, as demonstrated by the treatment de-escalation strategies employed in ACT3 and ACT4.

Many patients may accept a degree of acute and long-term morbidity if it means that they are cured of cancer. Unfortunately, the morbidity associated with LRF after CRT can be much more extensive. Salvage surgery is the primary rescue treatment for locoregional recurrence or persistent disease, usually abdominoperineal resection (APR). Pelvic exenteration may also be performed in carefully selected patients with more extensive disease. Although salvage surgery can result in long-term cure, outcomes are generally poor, with most studies giving a 5-year survival of around 40%[74–77]. Many studies do not report on morbidity at all, but those that do report 20-30% of patients develop major complications following surgery, defined as Clavien-Dindo 3-5, the majority of which are perineal wound complications. There is only one publication on long-term QoL following salvage surgery for ASCC, with 14 patients completing EORTC QLQ-C30 and EORTC QLQ-CR29 questionnaires. Most patients reported urinary frequency, with all male patients reporting impotence. Most patients reported fatigue, with half of patients reporting faecal incontinence, buttock pain, sore skin, flatulence, or stoma embarrassment[78].

Although salvage surgery is associated with significant morbidity, it is still the optimal treatment choice for patients with LRF. A study published 20 years ago in Manchester found that all patients with LRF who did not have salvage surgery died within 37 months[79]. Patients with LRF who are unsuitable for salvage surgery are currently enrolling in IO trials, which may become an alternative treatment option for LRF in the future.

1.1.12 Immunotherapy in metastatic anal cancer

Immune checkpoint inhibitors (ICIs) used as second-line treatment for metastatic or locally recurrent ASCC have had variable results, with objective response rates (ORR) ranging from 9-27% across several trials[80]. Selected patients from these trials have had sustained responses of up to 8 years. Although there is a trend for better response in PD-L1+ cancers in some trials, it is agreed that the current biomarkers cannot adequately identify patients most likely to benefit, with further translational studies needed. The InterAACT2 trial has finished recruitment for first-line chemo-immunotherapy combination using retifanlimab (PD-1 antibody) with carboplatin-paclitaxel. Early results were presented at ESMO2024, showing that the addition of retifanlimab increased median progression-free survival (PFS) (HR 0.63 [95% CI 0.47-0.84], median 9.30 vs 7.39 months, P=0.0006)[81]. The results of the complete publication and translational analysis are awaited.

Other areas of interest in ASCC IO have emerged outside of ICIs. High indoleamine 2,3 dioxygenase 1 was found to be a poor prognostic marker, and a trial of IDO1-targeted therapy has been suggested[82]. Metastatic HPV+ ASCC patients have been included in various tumour-agnostic trials for HPV+ cancer, such as bintrafusp alfa, a bifunctional fusion protein targeting Tumour growth factor-β (TGF-β) and PD-L1, and more recently, mRNA vaccine trials, but results for these are awaited[83,84].

1.1.13 Circulating tumour DNA and circulating tumour cell research in ASCC.

There are currently no validated blood-borne biomarkers to assist in clinical decision-making in ASCC, and circulating tumour DNA (ctDNA) is a promising new tool used in various solid tumours. Small fragments of cell-free DNA circulate in the bloodstream, giving timely information on molecular characteristics, disease status, and prognostic information[86]. ctDNA can be detected by measuring tumour-specific mutations or epigenetic alterations. Since the elimination half-life of ctDNA is short (<2 hours), biological clearance is expected immediately after curative treatment[87]. Data indicate that the presence of ctDNA in plasma post-surgery represents microscopic residual disease (MRD) and is known to imply an almost 100 % risk of recurrence and a poor outcome[87]. Most ctDNA studies report from CRC and lung cancer, but there is increasing interest in ctDNA analysis in SCCs. Notably, in HPV-associated cancers, HPV can be integrated into the tumour genome and consequently detected in the tumour DNA as a surrogate for ctDNA(Figure 1.5)[12,88]. The presence of circulating HPV-DNA (cHPV-DNA) in ASCC was confirmed in recent studies, indicating that up to 93% of cases

can be cHPV-DNA positive depending on the tumour stage and analytical method used[89]. A few studies have shown cHPV-DNA declines during CRT, and residual detectable cHPV-DNA after CRT seems associated with shorter disease-free survival (DFS) [90]. This has led to the initiation of a randomised Nordic trial prospectively investigating cHPV-DNA-guided follow-up[91]. A single study demonstrated three distinct elimination patterns during CRT and a high risk of failure in patients who did not obtain clearance during treatment[92]. In metastatic disease, persistent cHPV-DNA seems associated with poor prognosis after 1st line chemotherapy[93]. There are few published studies (<10), and the total number of patients included is limited.

Another blood biomarker is circulating tumour cells (CTCs). These are intact circulating tumour cells that have been shed from the cancer. They can provide cellular information to assess for druggable target mutations and be cultured for experiments. Although they provide more cellular and phenotypic information than ctDNA, they are less abundant and thus not as valuable for early detection of recurrence or monitoring during treatment. There is currently no published data on CTCs in ASCC.

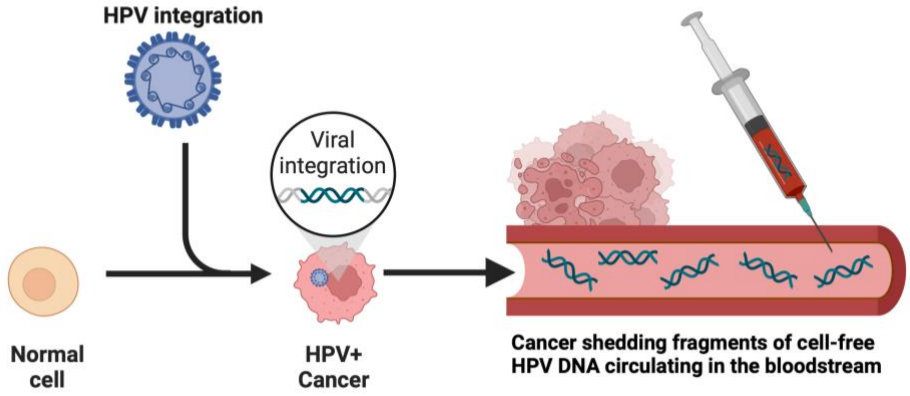


Figure 1.5 HPV integration into the tumour genome and subsequent cell-free DNA shedding allows HPV DNA to be used as a surrogate for circulating-tumour DNA

1.1.14 Available biological model systems of ASCC

HPV-associated cancers have pioneered biological models for cancer. Cancer cell lines were the first in-vitro cancer models produced, providing an indefinite source of biological material that is now fundamental for cancer research. The first human cell line was a CC HPV+ line, taken from Henrietta Lacks in 1951 and has been integral to many research advances, even being sent into space before manned missions to help understand the effects of radiation on future astronauts[94]. Since then, at least 52 CC lines and 73 HNSCC lines have been developed[95,96]. These cell lines have a vast array of characteristics – they can be HPV- or + for several HPV subtypes derived from primary or metastatic lesions, have different molecular profiles, and have different chemo- and radio-sensitivities. This array of characteristics allows researchers to answer specific

scientific questions for CC and HNSCC. In contrast, to our knowledge, the first 5 ASCC cell lines were developed in 2021[97].

This panel of cell lines was established from patients with both HPV+ and HPV- ASCC, including primary and relapsed disease. Whole exome sequencing revealed that these lines represent the ASCC patient population. They harbour many of the genomic aberrations frequently identified in ASCC. All lines were found to have strong tumorigenicity in a spectrum of immunocompetent and immunocompromised mice, with the histology of the xenografts demonstrating close recapitulation of the parent tumour. The panel of lines was assessed for cytotoxicity, with the two relapsed lines having substantial resistance to 5FU and all lines appearing to display resistance to MMC. A clonogenic RT assay confirmed the HPV- line to be the most radioresistant. A *PI3Kα*-specific inhibitor BYL719 (Alpelisib) was also assessed, with sensitivity demonstrated in four cell lines harbouring mutations or copy number gains at the *PIK3CA* locus.

The 2D growth of cell lines does not model the nutrient and oxygen gradient seen in tumours. Some of these cell lines can grow in 3D cultures known as spheroids that replicate this aspect of tumour heterogeneity and better mimic drug responses seen in the clinic. For CC, five cell lines, including HeLa, demonstrated chemotherapy responses closer to tumours for 3D models compared to 2D models of the same cell line, with ADAM17 identified as a novel target to overcome chemotherapy resistance[98]. For HNSCC, differences in radiobiological response between 2 HPV+ and HPV- spheroid models allowed for investigation of mechanisms of RT response[99]. Although technically challenging, immune cells can be added to spheroid cultures, allowing for the assessment of IO efficacy in CC cell lines[100].

ASCC tumoroids derived from cell lines have been described, with comparative histological features to the patient tumour from which they were derived. The tumoroids demonstrated modulation of PD-L1 expression in the presence of interferon-γ (IFN-γ), and three of the tumoroid lines were utilised in patient-matched TIL co-culture cytotoxic assays (unpublished data). This allowed assessment of TIL-directed killing, with the tumoroid line derived from a patient with a primary untreated cancer demonstrating pronounced cell death, compared with the two tumoroid lines derived from relapsed patients. Of note, adding an anti-PD-1 antibody to the assay did not alter the TIL-cytotoxic function in one of the relapsed tumoroid lines despite significant PDL1 expression.

Patient-derived organoids (PDOs) are another 3D tumour model generated directly from a patient's tumour tissue. They retain the tumour’s natural cancer cell genetic heterogeneity rather than just oxygen and nutrient gradients, better mimicking its pathophysiology. Spontaneously forming 3D structures based on their genetic programming allows for a better study of the TME. Numerous studies have been published on CC PDOs, which are utilised in precision medicine platforms and

identifying novel drug targets.[101,102] For HNSCC, PDOs have been developed that could be used for biomarker discovery and validation and assess response to novel therapeutics.[103,104] A panel of 7 human ASCC PDOs have been established in Australia, which recapitulates the morphological and histological characteristics of the parent tumour, have a genetic profile consistent with ASCC and are undergoing further characterisation (unpublished).

Traditional xenograft models are established from patient-derived tumour cell lines grown in vitro. In contrast, the term "Patient-derived xenograft" (PDX) is reserved for models that are established in immunocompromised rodents (typically mice) directly from a patient tumour biopsy or a surgical specimen. A major (theoretical) advantage of PDX models is that they bypass long-term culturing and associated risk for clone selection/enrichment and drift in gene- and phenotypes; they recapitulate the biology of the original tumours more faithfully, including the cellular and TME heterogeneity. Thus, PDX models may provide valuable information on patient biology, TME, biomarkers, and treatment sensitivity.

To our knowledge, minimal work has been performed on ASCC PDX models. The cell lines described above were used for the first example of an ASCC PDX model. As part of establishing the cell lines, when tumour biopsy material was limited, tumour tissue was initially expanded by implantation of biopsy material intramuscularly into severely immunocompromised mice, followed by the later establishment of cell lines from the xenografts. These PDX tumours were histologically and genetically similar to the patients from where they originated, and xenografts were reestablished from cell lines in culture. A Danish research group from Aarhus recently established 3 ASCC PDX models (unpublished, presented at IMAAC2023). Invasive analysis of administered hypoxia probes and hypoxia-regulated genes in an HPV+ and HPV- model revealed that both were profoundly hypoxic, which was in agreement with the patient tumours from where they originated (Figure 1.6)[12]. In contrast, at least 61 cervical-PDX models have been established.

Another ASCC mouse model in Australia has been established, based on a transgenic C57Bl/6 mouse with a tamoxifen-inducible Ubiquitin C-Cre-conditional knock-in of a *Pik3ca*^{H1047R} mutation and double deletion of *PTEN* (UBC-Cre.*pik3ca*^{H1047R}.*pten*$^{fl/fl}$ C57Bl/6) (Figure 1.7) (unpublished). Targeted application of 4-hydroxy Tamoxifen to the anal canal leads to the development of tumours. The tumours were confirmed to be invasive SCC and arise from the anal epithelium on histopathology. A syngeneic cell line has also been established from an ASCC of this mouse, with morphological and histological features consistent with ASCC. This line was transduced with human HPV 16 E6 and E7 oncogenes to create a separate line that recapitulated an HPV+ mouse ASCC. Both lines form tumoroids in an extracellular matrix, with similar histology to comparative human ASCC cell line tumoroids. They also demonstrate tumorigenicity in immunocompromised NSG and syngeneic wtC57Bl/6 mice, including growing orthotopically within the anal canal. A sub-clone of the E6/7 line has metastatic

potential, with pulmonary lesions confirmed to be SCC on histology. Therapeutic assays revealed the mouse lines to be more sensitive to 5FU but with a similar response profile to MMC as the human lines and greater resistance to RT. Assessment by flow cytometry of the immune infiltrate in the ASCC mouse cell line syngrafts demonstrated many similarities with the immune phenotype of human ASCC. A co-culture cytotoxic assay with syngraft TILs and mouse ASCC cell line tumoroids demonstrated limited killing, similar to the two human ASCC tumoroid lines developed from patients with relapsed disease.

Although this thesis does not attempt to develop new preclinical models of ASCC, it does put the research presented here in context. As discussed later in section 1.3.4, translating preclinical models to clinical trials presents many issues. This is particularly an issue for radiotherapy-immunotherapy (RT-IO) combination trials, where RT regimens and IO doses differ significantly from those used in the clinic. Developing translationally rich ASCC trials in the future requires knowledge of preclinical models and how these models can be used for reverse translation.

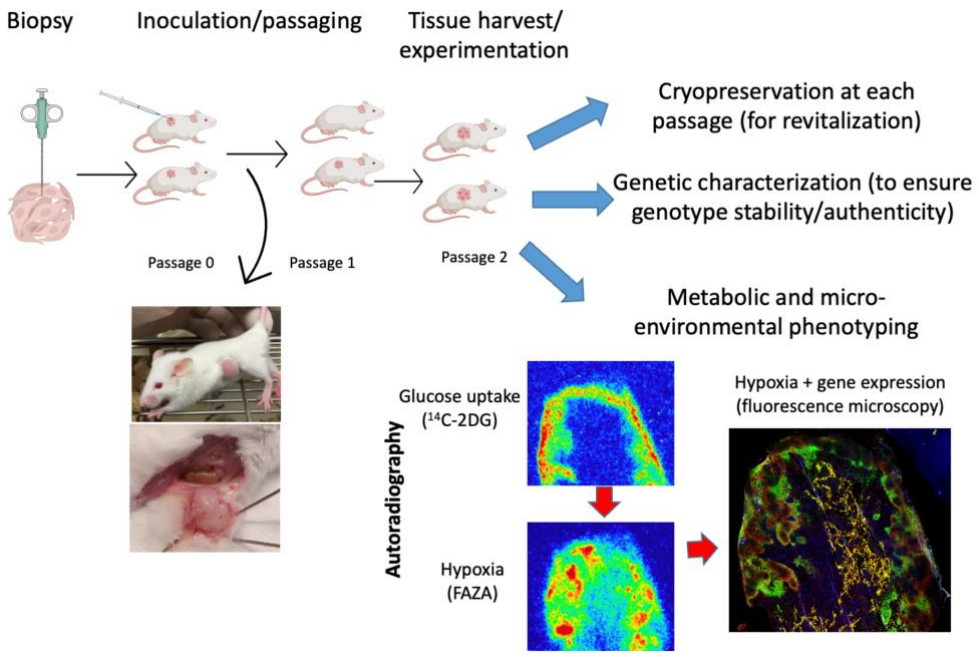


Figure 1.6 General workflow applied when establishing PDX models, including examples of analyses of TME and metabolism. Made by Morten Busk, Scientist at Aarhus University Hospital.

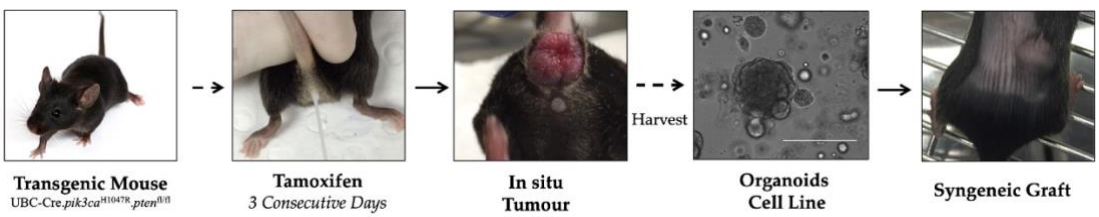


Figure 1.7 Development of ASCC following targeted 4-OHT application to the anal canal in UBC-Cre.pik3caH1047R.ptenfl/flC57Bl/6 mice. The subsequent tumour was then harvested for the development of a mouse ASCC cell line, which can be implanted as a syngraft in wtC5. Made by Glen Guerra, Surgeon at Peter MacCallum Cancer Centre, Melbourne.

1.1.15 Relevance of anal cancer to thesis

ASCC is an HPV-related cancer, with research and trials lagging more common HPV-associated cancers such as HNSCC and CC. Localised disease is treated with CRT, and although overall outcomes are good, individuals with locally advanced disease have a 25-30% chance of relapse. This group of patients require novel strategies to improve outcomes. Given HPV biology, the importance of the immune system in ASCC, and evidence from metastatic ASCC, IO may contribute to improving outcomes. cHPV-DNA is a promising tool for monitoring response to treatment. Although there are a burgeoning number of preclinical models of ASCC, none have directly assessed RT-IO combinations.

1.2 Radiotherapy and the Immune system

1.2.1 Overview of radiotherapy

RT is a cornerstone of cancer treatment, used in 50% of all cancer treatments and 40% of cancer cures[107]. It can be given externally using high-energy beams, usually photons, but also electrons, protons, and other heavy ions like carbon. It can be given internally as brachytherapy, either as seeds, ribbons, or capsules that can be placed permanently or temporarily inside the body. It can also be given systemically in the form of radionuclide therapy, most commonly Iodine-131 for thyroid cancer.

RT uses high doses of ionising radiation to kill cancer cells and works through a variety of mechanisms. It can cause direct damage to DNA or indirect damage via oxygen-free radicals that can induce single—and double-strand breaks. These breaks disrupt cell function and can lead directly to cell death or prevent cell replication. Given that DNA damage is the mechanism of action, cells in the G2 and M phases are the most susceptible, and as cancer cells are usually rapidly dividing, RT selectively kills a higher proportion of cancer cells than normal cells.

RT continues to undergo technical refinement. Advances in RT planning increase doses to the tumour while sparing normal tissues. However, it is close to the ceiling of toxicity, and previous attempts to add further drugs to treatment for ASCC have resulted in increased toxicity without improving outcomes[58]. Notably, the tumour remains in situ after definitive RT, with the immune system clearing ASCC over 3-6 months.

1.2.2 Overview of immunotherapy

In its most broad definition, IO is the treatment of disease by modulation of the immune system. In this broad definition, IO can suppress the immune system, including ciclosporin for organ transplants or infliximab for autoimmune conditions. In contrast, cancer IO activates the immune system to assist with recognising and then killing cancer cells. Evidence of the immune system's ability to treat cancer has existed since ancient Egypt, with reports of tumours disappearing after high-grade pyrexia. In the 19th century, William Coley began injecting different mixtures of live and inactivated bacteria into patients' tumours with reports of over 1000 remissions[108]. The understanding of the immune system evolved throughout the 20th century, particularly in the 1980s and 1990s with the discovery of checkpoint proteins such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and PD-1. Following their discovery, the first human trials of checkpoint inhibitors targeting these proteins were in the early 2000s. In 2011, ipilimumab was the first checkpoint inhibitor approved for advanced melanoma, followed by nivolumab in 2014. Since then, the number of indications and drugs targeting CTLA-4, PD-1, and its ligand PD-L1 has dramatically increased.

ICIs are the most widely recognised form of cancer IO today and are the focus of RT-IO trials, but there are many others. Chimeric antigen receptors (CAR) T-cell therapy uses genetically modified T-cells derived from the patient and engineered to target specific proteins on cancer cells. CAR T-cell therapy is more widely used in blood cancers than solid tumours, where delivery to the centre of the tumour mass and the TME pose additional challenges[109].

The rapid development of mRNA and other new vaccine technologies during the COVID-19 pandemic has sparked renewed interest in therapeutic cancer vaccines. There are many types of cancer vaccines - peptide-based, DC-based, DNA, RNA, and whole cell – but they all aim to present antigens that are overexpressed (tumour-associated) or unique (tumour-specific) antigens to the immune system, eliciting an initial response followed by immune memory. Although a burgeoning area in cancer research broadly, little has been published on RT-vaccine combinations.

1.2.3 Radiotherapy-induced immunogenic cell death and the cancer-immunity cycle

It is recognised that immune-mediated tumour cell death contributes significantly to the efficacy of RT[110]. Immunogenic cell death (ICD) is a process where the death of a cell elicits an immune response that can lead to the killing of other cells by the immune system. RT can support ICD through a few mechanisms. It can promote the release of damage-associated molecular patterns (DAMPs) such as calreticulin, high-mobility group Box 1 and heat shock proteins. RT also promotes the release of tumour-associated antigens (TAAs) through cell death and induces mutations within cancer cells that are then presented on major histocompatibility complex (MHC) molecules. DAMPs further stimulate interferons (IFNs) and inflammatory cytokine secretion, leading to enhanced activation and proliferation of T-cells targeting TAAs[111]. RT can

alter the TME by enhancing MHC expression on cancer cells and promoting inflammation by releasing cytokines such as IL-1 and TNF-α[112]. All the above can promote a process known as the cancer-immunity cycle (Figure 1.8)[113].

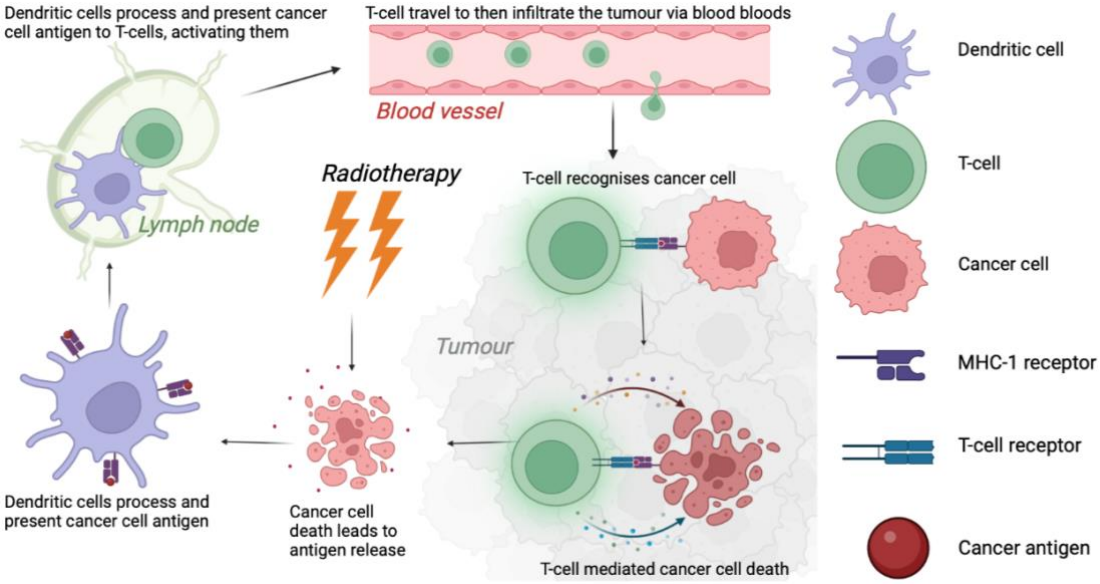


Figure 1.8 The cancer-immunity cycle is a cyclic process that amplifies and broadens T cell responses. Radiotherapy can promote the release of cancer cell antigens and increase the cancer-immunity cycle

1.2.4 Changes to tumour microenvironment

RT acting directly on immune cells can result in their activation inducing macrophage polarisation towards an M1 phenotype, maturating dendritic cells (DCs), and causing improved antigen presentation. It can upregulate NKG2D receptors on natural killer cells (NK cells) and cytotoxic CD8+ T-cells, increase TCR sequence diversity and increase MHC-I expression, all of which promotes direct cell-mediated killing. However, RT can also lead to immunosuppression. It can cause NK and DC inactivation, increase myeloid-derived tumour cell and tumour-infiltrating macrophage recruitment, and increase TGF-β secretion[114]. TGF-β has been shown to suppress CD4+ helper T-cells and support the maintenance of peripheral regulatory T-cells[115]. RT can also increase the expression of immune checkpoint proteins. All the above factors are likely to hinder RT immune-mediated efficacy[116].

1.2.5 cGAS-STING

In contrast to the cancer-immunity cycle, which connects the adaptive immune response with specific cancer antigens to RT, the cGAS-STING pathway exemplifies the connection between RT and the innate immune system. cyclic GMP-AMP synthase(cGAS) recognises DNA fragments in the cytoplasm. DNA fragments in the cytoplasm can be caused by viral infection, cellular damage, or RT. Upon binding to DNA, cGAS produces cyclic GMP-AMP, which then binds and activates the stimulator of

interferon genes (STING), producing IFN-β and initiating an immune response. Preclinical studies combined cGAS-STING agonists with RT have had positive results, with Phase I human trials ongoing[117,118].

1.2.6 Immune checkpoint proteins and response to radiotherapy

Immune checkpoint proteins are molecules expressed by cancer and immune cells that control immune system response. They are widely expressed in epithelial cells as a mechanism of peripheral tolerance. They can be co-stimulatory or co-inhibitory, providing opposing homeostatic regulation of the immune system. PD-1 and PD-L1 are two examples of co-inhibitory proteins that can be upregulated in cancer as a mechanism of immune evasion. PD-1 and PD-L1 are both overexpressed on cancer cells, which attaches to PD-1 or PD-L1 on immune cells in the TME. This binding can stop the immune cascade despite successful TCR-MHC binding and can lead to T-cell apoptosis. RT can increase PD-L1 expression in cancer cells through DNA damage signalling, IFN-γ signalling and cGAS-STING[119]. This is an example of immune resistance to RT and is the target of PD-1/PD-L1 inhibitors. To date, all approved RT-IO combinations are PD-1/PD-L1 inhibitors.

CTLA-4 is a co-inhibitory marker expressed on the surface of CD4+ and CD8+ T-cells. It competes with CD28, a co-stimulatory marker, to bind with CD80/CD86 on antigen-presenting cells, including cancer cells. The relative ratio of CD28/CTLA-4 binding to CD80/CD86 can dictate whether a T-cell becomes activated or inhibited when in contact with a cancer cell. Binding of CTLA-4 also leads to intracellular signalling that can inhibit TCR-MHC binding and the CD28 signalling pathway. In the TME, CTLA-4 can be overexpressed on T-cells, resulting in immune evasion. CTLA-4 is upregulated in response to RT. Ipilimumab, the first ICI approved in 2011 for melanoma, is a CTLA-4 inhibitor. CTLA-4-RT combinations have had mixed results. Boutros and colleagues found that in patients with melanoma, there was a 31% objective response rate for ipilimumab combined with RT. A single 9Gy dose was given 4 weeks after starting ipilimumab. Increased CD8+ cells in peripheral blood in response to RT were associated with better survival[120]. Demaria and colleagues found an objective response rate of 18% evaluable patients with chemo-refractory metastatic non-small lung cancer using ipilimumab combined with RT[121]. Two RT dosing schedules were chosen based on optimal murine models – 28.5Gy/3# and 30Gy/6#. Extensive translational samples from the patients suggest that patients with raised IFN-β and increased TCR expansion in response to RT had a better response[121].

PD-1/PD-L1 and CTLA-4 are the most common targets for RT-IO combinations with the most trial data published. However, based on preclinical data and evidence from trials without RT, RT-IO combinations for other immune checkpoint proteins are beginning to emerge. OX40 and CD137 are co-stimulatory proteins. They are expressed on T-cells only when activated and help to maintain an immune response. Their corresponding ligands, OX40L and CD137L, are expressed on antigen-presenting cells. Knisely and

colleagues performed a multi-cohort multi-arm that combined PD-1 inhibition with different combinations of RT, CD137 agonism and OX40 agonism[122]. Unfortunately, the results were poor, with an ORR of 2.9%. Most patients did not receive RT, and as commented by the authors, drawing conclusions for each target for individual cancers from this trial is difficult.

T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) is a co-inhibitory receptor upregulated on activated T-cells and NK cells. It binds to CD155 and CD112 on antigen-presenting cells. Binding to CD155 reduces TCR expression and decreases IL-12 secretion. Combining TIGIT and PD-L1 inhibition in PD-L1 positive non-small cell lung cancer (NSCLC) showed improved survival compared to PD-L1 inhibition alone[123]. This has led to the initiation of a multi-cohort trial investigating PD-L1/TIGIT inhibition with stereotactic ablative radiotherapy (SABR) in metastatic bladder cancer, HNSCC, renal cancer and NSCLC.

1.2.7 Cytokines in radiotherapy and immunotherapy

Cytokines are small soluble proteins that are important for signalling and cell communication. Once released by their cell of origin, they act on corresponding receptors on target cells. There are multiple classes of cytokines, including interleukins (IL), chemokines, growth factors and IFNs. They can be pro-inflammatory, driving innate and adaptive immune reactions, or mediating inflammation and repair. As discussed earlier, they can facilitate important immunological processes such as the cancer immunity cycle and the cGAS-STING pathway. Cytokines are, therefore, important for response to RT-IO combinations. Recombinant IL-2 and IFN-α were approved for monotherapies for cancer treatment in the 1990s with definite but limited clinical benefit. Following the success of ICIs, there has been renewed interest in targeting cytokines in combination with other therapies, including RT[124,125]

One example is granulocyte-macrophage colony-stimulating factor (GM-CSF) which can be used to avoid treatment-associated neutropenia and can promote DC function and immune response. An ORR of 16.7% was found when GM-CSF was combined with a PD-1 inhibitor and SABR for patients with metastatic chemo-refractory solid cancers[126]. Sequential flow cytometry showed that increased CD3+, CD4+, and CD8+ T-cells were associated with better response after one treatment cycle, but none were statistically significant. Cytokines IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were measured before and after treatment, but no relationship between cytokine changes and treatment response was found.

TGF-β is another cytokine of interest for RT-IO combinations. Although it inhibits the early stages of carcinogenesis, once cancer is established, TGF-β promotes cancer progression. TGF-β decreases cancer's sensitivity to RT, reduces CD8+ T-cell function, and promotes Treg function. To improve response in patients with locally advanced rectal cancer, TGF-β inhibition was combined with CRT[127].32% of patients had a complete response, defined as pathological complete response in patients who

proceeded to surgery, or clinical complete response maintained at 1 year after last therapy in patients with non-operative management. Although CD4+ and CD8+ cell populations decreased during treatment, there was no relationship between change in T-cell populations and response to treatment.

1.2.8 Approved RT-IO combinations

For both oesophageal cancer and locally advanced NSCLC, a significant and clinically meaningful increase in survival has been achieved with the addition of adjuvant ICIs after CRT, resulting in approval in the UK. The PACIFIC trial was a Phase III trial that randomised between durvalumab (PD-L1 inhibitor) and placebo in 713 patients with locally advanced, unresectable NSCLC who had not progressed after CRT. Patients with PD-L1 ≥1% had significantly longer PFS (HR 0.55 [95% CI 0.45-0.68], median 16.9 v 5.6 months) and overall survival (OS), (HR 0.72 [95% CI 0.59-0.89], median 47.5 v 29.1 months, p<0.001) with durvalumab compared to placebo[128]. The Checkmate 577 trial was a Phase II trial that randomised between adjuvant nivolumab (PD-1 inhibitor) and placebo in 794 patients with stage II or III oesophageal or gastroesophageal cancer who had residual disease after neoadjuvant CRT followed by surgery[129]. Patients who received nivolumab had significantly longer DFS (HR 0.69 [95% CI 0.56-0.86], median 22.4 vs. 11.0 months, p<0.001).

More recently, the KEYNOTE-A18 trial has led to approval in the USA of concurrent and adjuvant pembrolizumab (PD-1 inhibitor) in locally advanced CC. 1060 patients were randomised to pembrolizumab versus control, with PFS rates at 24 months of 68% in the pembrolizumab arm versus 57% in the placebo arm (median follow-up 17.9 months)[130].

1.2.9 RT-IO trials in other HPV-associated cancers

PACIFIC and Checkmate 577 have limited relevance to potential ASCC RT-IO combinations. This is due to the difference in biology and their RT schedules. In Checkmate 577, 11.6% of patients received <41.4Gy, 64% of patients received 41.4-50.4Gy, and 19.1% received >50.4Gy as part of neoadjuvant CRT *prior* to surgery. For ASCC, higher RT doses are used for *definitive* CRT. In contrast, although the dose of RT in the PACIFIC trial was closer to that used in locally advanced ASCC (92.9% received 54-66Gy), there is usually no elective nodal irradiation (ENI) for lung cancer RT plans. Checkmate 577 allowed local RT guidelines to be followed. Although oesophageal RT guidelines include ENI, the volumes are typically smaller than those of ASCC.

Due to patterns of relapse across HPV-associated cancers, RT plans include extensive ENI and are usually treated with definitive rather than neoadjuvant CRT. Although the results of KEYNOTE-A18 were positive, other published Phase III HPV-associated RT-IO trials have had less promising results. The phase III CALLA trial (NCT03830866) randomised 770 patients undergoing CRT (+ brachytherapy) for locally advanced disease to +/- concurrent durvalumab, with patients in the experimental arm

continuing adjuvant treatment for up to 2 years[131]. With a median follow-up of 18.5 months, the addition of durvalumab did not improve the primary outcome measure of PFS (HR 0.84 [95% CI, 0.65–1.08]; p=0.174).

Two large phase III trials evaluated the addition of ICI to CRT in patients with HNSCC and have reported primary outcomes. Javelin 100 randomised 697 patients undergoing radical CRT for locally advanced HNSCC to +/- avelumab (anti-PD-L1) with patients in the experimental arm receiving a single infusion pre-CRT, then concurrent Avelumab during CRT and continuing for 12 months[132]. There was no improvement in PFS, the primary outcome measure (hazard ratio 1·21 [95% CI 0·93–1·57] favouring the placebo group). Keynote 412 (NCT03040999) evaluated the same approach (neoadjuvant, concurrent, and then adjuvant), this time with pembrolizumab, again in patients with locally advanced HNSCC[133]. 804 patients were randomised between the two arms; with a median follow-up of 47.7 months (range, 37.0-61.4), the primary outcome measure of event-free survival (EFS) was not statistically different between arms (HR 0.83 [0.68-1.03], p=0.0429, significance threshold p<0.024), although the trend did favour the Pembrolizumab arm. In the cohort of patients with high levels of PD-L1 expression in the pre-treatment biopsy specimen (n=685), EFS just met significance (HR 0.80 [95% CI 0.64-1.00], p-value not given). Information on OS is awaited.

There are many other ongoing Phase III trials in HNSCC and CC (Table 1.1), and it is important to consider possible reasons why some of the published trials have not worked. Functional draining lymph nodes, RT volume and length, and biomarker selection are all possible explanations.

Functional draining lymph nodes are essential for priming T-cells against cancer antigens. Preclinical models suggest that RT to the draining lymph node dampens this adaptive immune response, reducing cytotoxic T-cell and DC signalling[134,135]. RT treatment of HPV-associated cancers typically includes elective doses to uninvolved locoregional lymph nodes and lymphatic drainage. ENI doses may be as high as 60Gy/30# for HNSCC in “intermediate risk” areas, which could impact anti-tumour T-cell priming. For ASCC and CC, RT volumes in the pelvis can be large, including para-aortic nodes and metabolically active bone marrow. The standard fractionation regime produces ongoing lymphopenia during and immediately after a 5–7-week treatment regimen. Given the inherent sensitivity of leukocytes to RT and the significant neutropenia and lymphopenia associated with pelvic irradiation, a careful re-analysis of the benefits and drawbacks of pelvic treatment volumes is required for RT-IO combinations. Any future studies in this area will require rigorous RT quality assurance (RTQA) to ensure lymph node doses are prescribed and recorded. A retrospective review of reported trials with high-quality RTQA such as JAVELIN may allow us to see if elective dose volumes have impacted IO efficacy.

Although survival stratified by PD-L1 expression was investigated in CALLA (secondary endpoint), Keynote 412 and JAVELIN (both exploratory), none of these trials used any

biomarker to select or stratify patient selection. Furthermore, PD-L1 positive definitions varied across all studies, making comparison difficult even within HNSCC - ≥25% tumour proportion score (TPS) in JAVELIN, ≥1% CPS in Keynote 412, ≥1% TPS in CALLA. Although outcomes vary for radical CRT across these cancers, certain groups of patients with locally advanced disease do well. Five-year LRF rates for patients with locally advanced ASCC are 20-30%. Therefore, three-quarters of unselected patients would get no benefit from IO whilst being exposed to potential side effects of additional treatment. This is also an issue for approvals. IO drugs are expensive, and improvements would have to be substantial in those that received a benefit for approval across non-selected locally advanced ASCC to be considered cost-effective.

The timing of IO in relation to CRT is another barrier to success, with discordant approaches across different preclinical models and clinical trials. Many mucosal SCC trials reference the same preclinical papers showing that concurrent or sequential IO (starting within seven days of finishing RT) was superior to adjuvant IO[42,136]. However, many of these trials then investigated combinations of neoadjuvant, concurrent and adjuvant regimens, and have taught us little about the optimal scheduling of ICIs.

Table 1.1 Ongoing Phase III trials in other HPV-associated cancers investigating RT-IO combinations.

Trial identifier	Title	Inclusion criteria	Immunotherapy	Immunotherapy regime in relation to CRT	N	Estimated completion
Cervical						
NCT05173272	Induction Chemotherapy Combined With Immunotherapy Followed by Concurrent Chemoradiation in Advanced Cervical Cancer	Cervical Cancer FIGO 2018 Ib3-IIIc2	Anti-PD-1 Slulimumab	Neoadjuvant with chemotherapy	286	28/12/2028
NCT05235516	A Study of AK104/Placebo Combined With Chemoradiotherapy For The Treatment of Locally Advanced Cervical Cancer (AK104-305)	Cervical Cancer FIGO 2018 Stage IIIA-IVA	Anti-PD-1/CTLA-4 bi-specific antibody Cadonilimab	Concurrent	636	01/05/2029
NCT04221945	Study of Chemoradiotherapy With or Without Pembrolizumab (MK-3475) For The Treatment of Locally Advanced Cervical Cancer (Keynote A-18)	Cervical Cancer FIGO 2014 Stages IB2–IIB with N+ or III–IVA	Anti-PD-1 Pembrolizumab	Concurrent and adjuvant	980	07/12/2024
HNSCC						
NCT03765918	Study of Pembrolizumab Given Prior to Surgery and in Combination With Radiotherapy Given Post-surgery for Advanced Head and Neck Squamous Cell Carcinoma (MK-3475-689)	Stage III OP HPV +ve cancer, Stage III/IVA OP HPV negative cancer, Stage III/IVA larynx/HP/oral cavity cancer	Anti-PD-1 Pembrolizumab	Neoadjuvant and concurrent	704	30/07/2026

Trial identifier	Title	Inclusion criteria	Immunotherapy	Immunotherapy regime in relation to CRT	N	Estimated completion
NCT03576417	A Trial Evaluating the Addition of Nivolumab to Cisplatin-RT for Treatment of Cancers of the Head and Neck (NIVOPOSTOP)	Oral cavity, OP, HP, or larynx cancer with high risk of relapse	Anti-PD-1 Nivolumab	Neoadjuvant, concurrent and adjuvant	680	01/09/2027
NCT03952585	De-intensified Radiation Therapy With Chemotherapy (Cisplatin) or Immunotherapy (Nivolumab) in Treating Patients With Early-Stage, HPV-Positive, Non-Smoking Associated Oropharyngeal Cancer	p16 +ve T1-2N1M0 or T3N0M0 OP cancer	Anti-PD-1 Nivolumab	Neoadjuvant, concurrent and adjuvant	711	28/02/2025
NCT03700476	Sintilimab (PD-1 Antibody) and Chemoradiotherapy in Locoregionally-advanced Nasopharyngeal Carcinoma (CONTINUUM)	Stage III/IVA Nasopharyngeal Cancer	Anti-PD-1 Sintilimab	Neoadjuvant, concurrent and adjuvant	425	01/0125
NCT01810913	Testing Docetaxel-Cetuximab or the Addition of an Immunotherapy Drug, Atezolizumab, to the Usual Chemotherapy and Radiation Therapy in High-Risk Head and Neck Cancer	Stage III/IV p16 negative oral cavity, OP, larynx, HP cancer	Anti-PD-L1 Atezolizumab	Neoadjuvant, concurrent and adjuvant	613	01/01/2027

Trial identifier	Title	Inclusion criteria	Immunotherapy	Immunotherapy regime in relation to CRT	N	Estimated completion
NCT03258554	Radiation Therapy With Durvalumab or Cetuximab in Treating Patients With Locoregionally Advanced Head and Neck Cancer Who Cannot Take Cisplatin	Stage III p16 +ve OP/SCC of unknown head/neck primary cancer or Stage III-IVB p16 –ve laryngeal, HP, and oral cavity cancer	Anti-PD-L1 Durvalumab	Concurrent and adjuvant	493	31/12/2025
NCT02999087	Randomized Trial of Avelumab-cetuximab-radiotherapy Versus SOCs in LA SCCHN (REACH)	Stage III/IVA/IVB Oral cavity, OP, HP, or larynx cancer	Anti-PD-1 Avelumab	Concurrent and adjuvant	707	01/12/2027
NCT03427827	PD-1 Antibody Versus Best Supportive Care After Chemoradiation in Locoregionally Advanced Nasopharyngeal Carcinoma (PACIFIC-NPC)	Stage III/IVA Nasopharyngeal Cancer	Anti-PD-1 Camrelizumab	Adjuvant	442	01/02/2026
NCT03452137	A Study of Atezolizumab (Anti-Pd-L1 Antibody) as Adjuvant Therapy After Definitive Local Therapy in Patients With High-Risk Locally Advanced Squamous Cell Carcinoma of the Head and Neck	Squamous Cell Carcinoma of the Head and Neck, not nasopharynx or paranasal sinuses	Anti-PD-L1 Atezolizumab	Adjuvant	406	01/06/2027
NCT03700905	Study of Nivolumab Alone or in Combination With Ipilimumab as Immunotherapy vs Standard Follow-up	Stage III-IVB HPV negative OP, oral cavity, HP, and larynx cancer	Anti-PD-1 Nivolumab,	Adjuvant	276	01/05/2024

Trial identifier	Title	Inclusion criteria	Immunotherapy	Immunotherapy regime in relation to CRT	N	Estimated completion
	in Surgical Resectable HNSCC After Adjuvant Therapy (IMSTAR-HN)		Anti-CTLA4 Ipilimumab			
NCT03811015	Testing Immunotherapy Versus Observation in Patients With HPV Throat Cancer	p16 positive OP cancer	Anti-PD-1 Nivolumab	Adjuvant	636	01/01/2027

1.2.10 Ongoing RT-IO trials in anal cancer

Given its rarity, there is less research on ASCC compared to other HPV-associated cancers. Consequently, fewer RT-IO trials have been initiated, and only two have reported preliminary results[132,133,137–139]. These trials will be discussed in-depth in Chapter 5 as part of a systematic review. Knowledge of specific aspects of how these trials are designed, such as optimal RT fields, choice and immunotherapeutic drug(s) schedules, and patient selection, are needed to enable us to design future trials accordingly [140]. The biological basis for ASCC-specific IO integration with RT and translational analysis plans are examined as essential components for all future ASCC RT-IO trials.

1.2.11 Relevance of radiotherapy and the immune system to thesis

There are multiple mechanisms by which RT and the immune system interact. Each of these mechanisms is a potential target for drug therapy. Although there are approved RT-IO combinations that have changed the standard of care in some settings, their relevance to ASCC is limited. Results from other HPV-associated trials have been mixed, with no ASCC trials yet reported.

1.3 Clinical Trial Methodology

1.3.1 Introduction

To effectively address the most important research questions, it is crucial to use clinical trial methodology that is robust, reliable, and valid. Trials need to be applicable to their target population whilst being reproducible and exhibiting statistical rigour. Due to the increasing costs of running trials, particularly in specific patient populations, and the growing availability of new drugs and technologies to investigate, it is important to consider alternative approaches to traditional randomised parallel group designs. Master protocols and adaptive designs are examples of trial methodology advancements pertinent to RT-IO trials.

The first and second generations of ASCC trials, discussed in 1.1.9, demonstrate the importance of clinical trial methodology. Heterogeneity in many aspects of trial design, including outcome selection and definition, has resulted in global differences in the standard of care for localised ASCC. Utilising a core outcome set (COS) when designing the next generation of personalised drug-CRT combination trials in ASCC will improve between-study comparisons, increasing the likelihood that patients outside of clinical research will ultimately benefit from improvements in care.

1.3.2 Master Protocols

There is increasing use of innovative master protocol trial designs in cancer trials. Master protocol trials are developed to simultaneously evaluate more than one intervention and/or multiple different subpopulations within the same overall trial

protocol, offering the opportunity to expedite treatment development processes. The efficiencies of this approach have been recognised and employed in trials of COVID-19 agents[141]. Master protocols may be categorised into basket, umbrella, and platform[142]. Basket designs refer to a trial whereby a targeted therapy is evaluated within multiple disease types with a common molecular characterisation or similar underlying patient characteristics. Basket trials enable the investigation of a single molecular characteristic across various tumours, allowing for comparison of the effectiveness of targeting that characteristic in each disease site. NCT02454972 is an example of a Phase II basket trial investigating lurbinectedin, a selective inhibitor of oncogenic transcription, across nine different cohorts of patients - relapsed small cell lung cancer, HNSCC, neuroendocrine tumours, biliary tract carcinoma, endometrial carcinoma, BRCA 1/2-associated metastatic breast carcinoma, carcinoma of unknown primary site, germ cell tumours and Ewing's family of tumours. This was much more efficient than running 9 “single-arm” trials[143–145].

Umbrella designs refer to a trial evaluating multiple therapies within a single disease type, incorporating treatment stratification by, for example, molecular characterisation. The National Lung Matrix Trial is a large umbrella trial that uses next-generation sequencing to screen for 28 genes in patients with advanced NSCLC after progression on first-line chemotherapy. There are 22 treatment arms across eight target therapies, with an additional arm for patients with no actionable mutations[146]. By tailoring treatment to specific molecular characteristics, umbrella trials provide treatment personalised to an individual’s cancer. Running one large umbrella trial can be cost-effective compared to multiple small trials investigating a single characteristic.

Platform trials provide a flexible protocol that evaluates multiple experimental treatments (more than two) for a single disease type. They are adaptive, incorporating predefined rules allowing the addition or removal of treatments during the trial. For example, if a new biomarker or treatment emerges after the trial begins, it can be integrated subsequently. They usually include randomisation to a control arm. The FRACTION-Lung (Fast Real-time Assessment of Combination Therapies in Immuno-Oncology) is a platform trial designed by Bristol-Myers Squibb, which allows for new combination IO regimens to be added to the study as they become available and ineffective regimens are withdrawn. This approach enables patients who are not responding to an initial regimen to be re-randomised to a new arm[147]. Platform studies are helpful when patients have more than one treatment option.

These types of master protocols can be combined. For example, stratification within platform trials may result in umbrella platform trials, whereby multiple experimental treatments may be considered within each stratified group of patients. Here, a control arm may be included for each stratified patient group of interest. Platforms can also be stratified by biomarker or disease type. The FRACTION-Lung platform trial is one stratum of the overall FRACTION platform, which assesses multiple tumour sites: FRACTION–lung, FRACTION-RCC, and FRACTION-gastric cancer.

The examples of master protocols described concern trials investigating drug therapies[148,149]. However, there are additional specific benefits for master protocols in RT-focused clinical trials, where novel technology, treatment personalisation, and RT dose or novel RT-drug combinations may be evaluated. The SMART (Stereotactic Magnetic Resonance Guided Adaptive Radiation Therapy) trial is an example of a basket trial investigating a new RT technology. SMART is investigating MR-Linac technology across multiple types of tumours within one trial, aiming to identify areas where MR-Linac technology could be beneficial compared to standard RT[150]. Given the high upfront capital costs associated with new RT technologies, knowing which indications may improve outcomes is essential. Proton therapy is an example of a technology that was widely adopted in the USA without strong evidence for prostate cancer, leading to financial challenges for many proton centres when insurance providers decided not to cover treatment[151]. As discussed earlier, the PLATO trial is an example of an umbrella trial[72]. The trial has five arms, each delivering a different RT dose, with eligibility to each arm dictated by disease stage. CONCORDE is a Phase I platform trial evaluating DNA damage response inhibitors in combination with RT for patients with locally advanced NSCLC who are not fit for CRT[152]. One of its 5 arms is a control arm, receiving RT only allowing for the assessment of dose-limiting toxicities across arms of the platform.

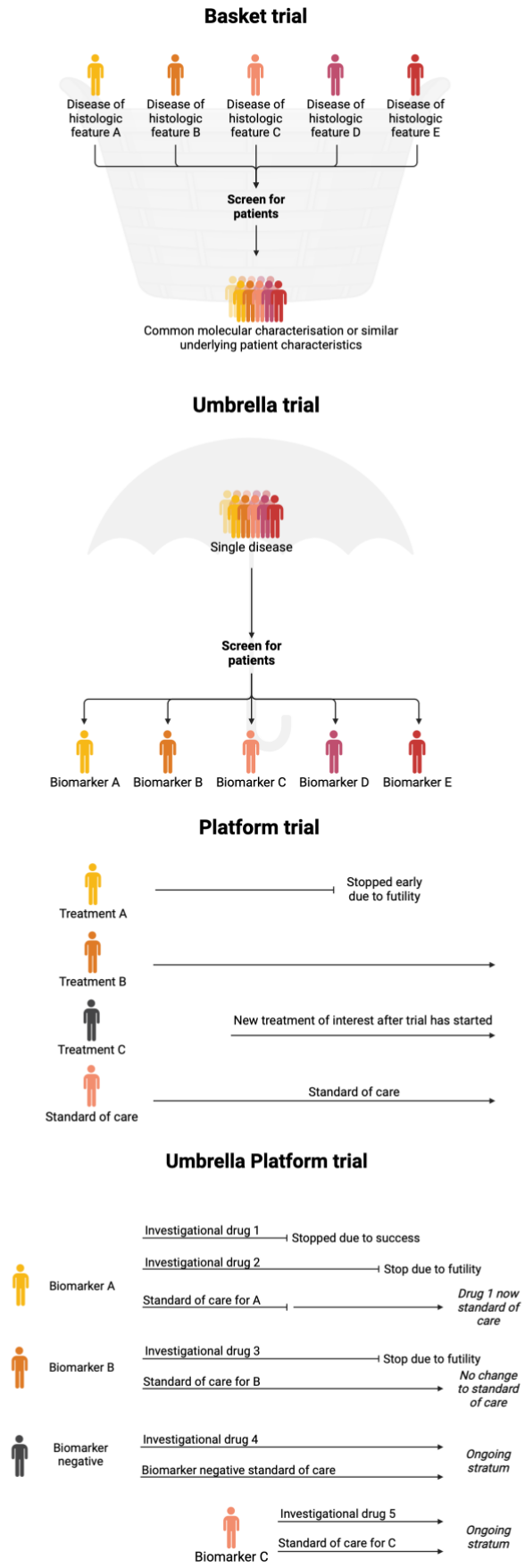


Figure 1.9 Different master protocol trial designs.

1.3.3 Adaptive trial design

Adaptive trial designs allow pre-specified modifications based on interim analysis results during an ongoing trial. Compared to traditional, fixed clinical trials, they allow for increased flexibility. Modifications can apply to aspects such as sample size, enrichment, dropping treatment arms, drug dosing, and changing the allocation ratio of patients to trial arms[153]. In a standard two-arm adaptive enrichment trial, patients will be recruited with or without a biomarker of interest and randomised to either the experimental or control arm. Following interim analysis, recruitment may not be adapted at all, be adapted to recruit only those with the biomarker, only those without the biomarker or the study may be terminated for futility. If the biomarker of interest is not naturally dichotomous, the same interim analyses may also be used to select or revise marker cutpoints. For example, this approach could be used for PD-L1% expression cut-offs in an RT-IO trial. The MICRO trial investigated ontuxizumab in metastatic colorectal cancer. This drug targets endosialin function and has several potential continuous biomarkers. The interim analysis compared the results of the whole population and assessed the optimal cut-off for the biomarkers. This analysis showed no predictive signal for any of the biomarkers and found no benefit in the whole population compared to placebo, and the trial was terminated[147].

A “Seamless” trial is an example of adaptive design combining different clinical trial stages. An interim analysis is performed after each stage before the next one starts, without setting up a new trial. For example, in seamless Phase I/II trials, safety data and dose finding from a new treatment in Phase I is followed by investigating its efficacy in Phase II. NCT02444741 is an example of a RT-IO Phase I/II trial that investigated the addition of pembrolizumab to RT (either 50Gy/4# or 45Gy/15#) for metastatic NSCLC[154]. In the Phase I portion, a traditional 3+3 dose-escalation design was used for pembrolizumab when combined with RT. Once a dose of 200mg of pembrolizumab was reached in Phase I, this was used in the Phase II portion for a 1:1 randomisation to RT or no RT. In oncology, Phase II/III trials are often “inferentially seamless”, combining efficacy data from the Phase II trial into the Phase III cohort that may be used for regulatory approval. Alliance A082002 is an example of a RT-IO Phase II/III trial, comparing SABR (8Gy/3#) plus chemo-immunotherapy versus chemo-immunotherapy alone for NSCLC[155]. The primary endpoint of the Phase II trial is PFS, which will recruit 100 patients. The primary endpoint of the Phase III trial is OS, which will recruit a further 284 patients. In this trial, OS in the Phase II cohort can be combined with the Phase III cohort, reducing the required sample size. Phase II/III seamless can be challenging in settings where the phase II endpoint takes a long time or is difficult to observe before deciding whether to proceed to phase III.

In contrast to a master protocol approach, which refers to how a protocol is set up, adaptive trial design is a broad term that refers to the statistical method used for different comparisons within a trial. Therefore, a trial can be both a master protocol and have an adaptive design. Seamless design, adaptive design and master protocols

are combined in multi-arm, multi-stage (MAMS) trials. Multiple different treatments can be compared, and with predetermined interim analysis, these can be modified, dropped if showing signs of futility, or closed early if they show better than expected efficacy. Although initially more expensive and complex to set up, MAMS can answer multiple clinical questions simultaneously under a single regulatory framework. As ineffective arms can be dropped and more promising treatments promoted, patients are more likely to be recruited for beneficial treatment. STAMPEDE is an example of a large MAMS trial in prostate cancer that recruited almost 12000 patients across the UK from 2005-2023[156]. Its broad aim was to find the best way of treating men with newly diagnosed advanced prostate cancer, initially starting with five arms, including the addition of docetaxel to long-term hormone therapy, which became the new standard of care. Since then, multiple arms of the study have been evaluated in different subgroups of patients, including those with locally advanced prostate cancer and low-burden metastatic disease.

1.3.4 Specific trial methodology considerations for new RT-drug combination trials and preclinical studies

Notwithstanding concurrent chemotherapy, RT-drug combinations are rarely used in routine clinical care despite promising results from preclinical studies[157,158]. This is not just an issue for IO, DNA damage inhibitors are another example with a clear scientific rationale of synergy with RT that have yet to make the jump to routine care[159]. There are specific considerations for new combination studies of radiation therapy and drugs that make their design more complex and may be one of the reasons for the lack of translation from preclinical evidence into patient benefit.

The choice of RT regimen for novel RT-drug combinations can be difficult, and interpreting the optimal RT dose and fractionation from these preclinical studies has several issues. A 5-7 week course of daily fractionated 1.8-2Gy per day is the standard curative RT regimen for many cancers, and many RT-drug trials will use this regimen. The preclinical models on which these are based often use much smaller total doses, usually over a shorter time. This may cause the synergy between the drug and RT in the preclinical model to be lost and explain why these trials are unsuccessful.

These standard RT regimens are based on decades of clinical evidence. Generating enough evidence to convince oncologists and ethics boards to adopt experimental RT regimens is a challenge. Designing studies to create this evidence base requires consideration of how the response to RT is monitored in real-time. One option could be to use blood biomarkers of response to shorten the treatment of patients who demonstrate a “complete biological response” during standard fractionated RT. For example, if there was no detectable cHPV-DNA halfway through standard RT for ASCC, treatment could be stopped, and cHPV-DNA monitored. Another option is to investigate these drugs in patients where high-dose palliative treatments are planned. 30Gy/10# or 20Gy/5# are standard regimens used for symptom control for multiple

tumours that more closely mimic preclinical regimens. Good efficacy in these studies would support these regimens being used in the curative setting. When RT is not part of routine clinical care, it is easier to justify using regimens closer to those in preclinical models. For example, the CTLA-4-RT combination study in palliative lung cancer by Demaria and colleagues used the two best RT regimens evaluated in murine models before the human Phase I trial[160].

Another issue in RT-drug combination trials is the quality and reproducibility of preclinical data. In 2016, Stone and colleagues collected data from 125 published papers concerning the interaction of 10 drugs with radiation[161]. It found mixed results for 9 of the 10 drugs, with sunitinib ineffective in all but one study. A wide range of radiation doses and drug concentrations were used, with 83% of studies having problems that would make replicating the experiment difficult. They proposed a checklist for preclinical studies evaluating RT-drug combinations focused on detailed reporting. Although none of the drugs investigated were IO, many of the same principles apply. One area of difference for IO is the choice of assays used to measure effectiveness. Most other radiation modifiers work by directly increasing the effectiveness of RT to reduce the survival and proliferation of cancer cells. The gold standard for measuring this is the clonogenic assay. As IO indirectly enhances this ability and requires the presence of immune cells, this gold standard assay is not feasible, making preclinical comparisons in IO harder than other potential radiosensitisers.

Compared to standard early-phase drug-only trials, interpreting dose-limiting toxicities in RT-drug trials is difficult given the expectation of grade 3 toxicity during standard definitive RT treatment and the extended period for late RT toxicities, which often occur at least a year following RT. These toxicities apply to the target organ and OARs within the treatment field[162]. The toxicity profile in RT-IO treatment is different to other drugs used in combination with RT, with IO also associated with long-term toxicities that can occur after finishing treatment. When used in isolation, the toxicities observed in RT and IO overlap. For example, in a RT-IO trial in lung cancer, determining whether RT alone caused pneumonitis, IO alone caused pneumonitis, or the synergistic effect of both treatments caused pneumonitis would be challenging. Fibrosis induced by RT is the process most associated with long-term RT toxicities and is influenced by the immune system. This complex interaction needs to be better understood and requires further preclinical study[163]. Designing trials to evaluate long-term toxicities and aid attribution is essential[164].

1.3.5 Reverse translation and its importance in rare cancers

In clinical trials, reverse translation refers to transferring findings from clinical research (*benchside)* to basic science to improve biological understanding (*bench*). This understanding then informs future research, including generating new hypotheses and

developing new treatments. It can start an iterative process that drives research questions and improves outcomes (*bench to bedside and back again*).

Compared to more common cancers such as breast cancer and colorectal cancer, reverse translation is even more critical in rare cancers such as ASCC. There are fewer patients to participate in clinical trials and often fewer preclinical disease models. Therefore, more data is needed from each patient to improve understanding of ASCC cancer biology. Approvals for new treatments are more challenging in rare cancers due to the difficulty of running large Phase III trials. As a result, rare cancers often have comparatively limited treatment options[165,166]. Rare tumours can also be common tumours with rare mutations, of which there are a few examples of well-designed translational analyse from Phase I/II basket trials that have led to approvals in specific rare indications[167,168].

1.3.6 Core outcome set

A COS is a standardised set of outcomes that should be measured and reported at a minimum in all trials of a specific clinical area[169]. Organisations like NICE, regulators such as the FDA and EMA, and trial funders such as Cancer Research UK and NIHR actively encourage the adoption and use of COS.

While the specific methodology may differ for each COS, the process is generally consistent. Initially, information on all potential outcomes is collected, which may include a systematic review of the literature and semi-structured interviews with patients and clinicians to create an extensive list. Afterwards, this list can be analysed to combine closely related outcomes.

The second stage is to hold a consensus process to agree on the COS. To ensure validity and encourage participation, clinicians and patients are usually involved. The Delphi method is the most used consensus method to ensure that all participants' views are heard[170]. Other methods, such as the nominal group technique, are also used[171]. In the context of a COS, the Delphi method involves sequential rounds of anonymous voting on each outcome, with prespecified criteria for inclusion or exclusion. After the first round, participants can suggest additional outcomes or clarify issues for each outcome. This can be an iterative process across more than one round. Following the last round, a consensus meeting is held where outcomes that have reached prespecified criteria are ratified and outcomes where there is no consensus are discussed before further voting, eventually resulting in an agreed COS. Usually, a COS is a recommendation of "*what*" should be reported. However, the last step of COS development, often not performed by COS researchers, is "*how*" each outcome should be defined or measured, including which instruments should be used.

1.3.7 CORMAC

The six Phase III randomised trials for ASCC, detailed in 1.1.9, defined the current standard of care and provide a good example of the issue that COSs seek to address.

Variations in primary and secondary outcomes, as well as how the primary outcome was defined, hindered evidence synthesis from these trials (Table 1.2). The Core Outcome Research Measures in Anal Cancer (CORMAC) project was the first COS for ASCC. It was designed to be used in trials involving CRT to treat ASCC. Following the Core Outcome Measures in Effectiveness Trials (COMET) methodology, results of a systematic review and qualitative patient interviews were combined for a list of outcomes[172,173]. The systematic review identified 95 eligible studies identified that reported 1192 different outcomes with 533 unique terms. These were collapsed into 86 standardised outcomes across five domains: survival, disease activity, life impact including quality of life, delivery of care, and toxicity. These were combined with outcomes identified from interviews with patients who have previously had CRT for ASCC. Following this, a 2-stage Delphi process was completed, followed by a consensus meeting of healthcare professionals and patients. This resulted in a COS with 19 outcomes across four domains: disease activity, survival, toxicity, and life impact. The next step of the CORMAC project is to agree on how these outcomes should be defined, which will performed as part of this thesis.

Table 1.2 Primary and secondary outcomes in Phase III randomised trials of CRT. Adapted from Fish et al. 2018[172]

Trial, year of publication	Local treatment failure	Progression-free survival	Disease-free survival	Colostomy-free survival	Colostomy	Acute toxicity	Overall survival	Cancer-specific survival	Local/regional control
ACT I (1996)	✓[1]			♦			♦	♦	
RTOG 87-04 (1996)	✓[2]		♦	♦	♦	♦	♦		♦
EORTC (1997)	✓[3]				♦	♦	♦		
RTOG 98-11 (2008)			✓		♦	♦	♦		♦
ACCORD-03 (2012)				✓			♦	♦	♦
ACT II (2013)	✓[4]	✓		♦		✓	♦	♦	♦

✓Primary outcome; 1, clinically, at 6 weeks; 2, on biopsy, post-irradiation; 3, clinically, at 6 weeks; 4, clinically, at 26 weeks

♦ Secondary outcome

Table 1.3 CORMAC domains and outcomes

Domain	Outcomes
Survival	Overall survival Cancer specific survival Disease-free survival Metastasis-free survival Progression-free survival
Disease activity	Treatment response Local failure Regional failure Distant failure Disease progression
Toxicity	Anal incontinence Faecal urgency Pelvic fistula Colostomy/ileostomy Skin blistering/sloughing
Life impact	Physical function Sexual function Health related quality of life

1.3.8 Relevance of clinical trial methodology to thesis

Aspects of clinical trial methodology, such as master protocols and adaptive trial design, should be appropriately used to improve the quality of future RT-IO combination trials. High-quality translational analysis is essential, given the poor understanding of ASCC biology and the limited number of patients available for clinical trials. A systematic review of all RT-IO ASCC trials will examine what has already been performed to assist the design of a future clinical trial. The CORMAC project demonstrates heterogeneity in the ASCC trials that defined the current standard of care. Although a COS has been developed, the outcomes have yet to be defined and will be addressed in this thesis.

1.4 Project rationale and aims

As discussed earlier, ASCC is a rare HPV-driven cancer with an increasing incidence globally. Patients with locally advanced disease have a 25-30% chance of treatment

failure. We need alternative treatment strategies to lower this failure rate, and one option could be a combination of RT and IO. While there are examples of RT and IO combinations changing the standard of care, such as PACIFIC and Keynote412 in NSCLC and oesophageal cancer, these have limited relevance to ASCC. Early results from trials in other HPV-associated cancers, such as JAVELIN100 and CALLA, have been disappointing, and there are few preclinical models specific to ASCC that we can use to guide a future RT-IO ASCC trial.

My research aims to:

i) Identify immune resistance markers in standard of care CRT for locally advanced ASCC by evaluating samples taken before, during, and after. Different laboratory techniques are used to investigate the cellular (Chapter 3) and soluble markers (Chapter 4) of immune resistance.

ii) Evaluate and propose approaches to trial design and methodology in a new RT-IO ASCC trial context. “Rationally developing” such a trial refers to a systematic and evidence-based approach to maximise the likelihood of trial success. Trial methodology is an integral part of this process. In Chapter 5, a systematic review of all current ASCC RT-IO trials will be performed, and the second stage of the CORMAC COS will be developed.

1.4.1 Aim 1

To identify pre-CRT and CRT-induced cellular markers of ASCC immune resistance and to correlate these with 6-month treatment failure (circa 25-30%) and complete response (circa 70-75%).

1.4.2 Aim 2

To identify pre-CRT and CRT-induced soluble markers of ASCC immune resistance and to correlate these with 6-month treatment failure (circa 25-30%) and complete response (circa 70-75%).

1.4.3 Aim 3

To rationally inform and assist novel early-phase clinical trial design to integrate IO into future curative ASCC trials.

Chapter 2 Material and Methods

2.1 Ethical Permissions

Patients were recruited from three large, research-active centres in the United Kingdom to ensure the collection of sufficient samples within a timeframe that allowed laboratory processing and analysis. All patients were prospectively recruited.

2.1.1 Leeds ethical permissions

Patients were recruited at St. James' Hospital using "Preclinical Assessment of Anti-Cancer Cellular Vaccines and Therapies to Stimulate Anti-tumour Immunity" 06/Q1206/106. I recruited the patients with assistance from Beccy Smith, a Senior Clinical Trials Assistant in Radiotherapy. Fay Ismail helped me process the samples.

2.1.2 Manchester ethical permissions

Patients were recruited at the Christie Hospital using the Manchester Cancer Research Centre Biobank ethics. The lower GI Clinical Oncology clinicians recruited patients – Professor Mark Saunders, Dr Noor Alam, and Dr Peter Mbanu. The lower GI research team performed coordination of sample collection and data handling – Anup Shanthappa, Lilly Simpson, and Jess Dyke. Blood samples were processed by the Clinical Research Facility at the Christie Hospital. Samples were processed following the methods below by the Manchester Clinical Research Facility.

2.1.3 Oxford ethical permissions

Patients were recruited at the Churchill Hospital using the Oxford Radcliffe Biobank ethics. Dr Rebecca Muirhead recruited patients. As part of a research collaboration, Monica Olcina, David MacLean, and Dominika Majorova processed the blood samples following the methods below.

2.2 Sample collection

The sample collection and laboratory analysis were collectively called GRECIAN (identifyin**G R**adiotherapy Immune r**E**sistance me***C***hanisms **I**n **A**nal cancer) as this work relates to the PLATO trial, and GRECIAN means pertaining to ancient Greek.

2.2.1 Target patient population

Patients with locally advanced ASCC, defined as T3-4NanyM0 or TanyN1M0, were eligible for recruitment. Only patients fit enough to receive full-dose CRT were included. At the time of GRECIAN recruitment initiation, ACT5 recruitment was ongoing. The eligibility criteria for ACT5 are very similar to GRECIAN. Due to the small number of eligible patients within each site, it was decided that patients could be enrolled in both ACT5 and GRECIAN.

2.2.2 Classification of complete response and treatment failure

As part of their routine clinical follow-up at these three centres, all patients receive an MRI scan and PET-CT scan at 3 months and 6 months after completion of CRT. ASCC response to CRT can take several months, and patients can have a partial response (as per RECIST criteria) at their 3 month scan that becomes a complete response at their 6 month scan. Although more unusual, if there is low uptake on PET-CT and clinical exam is not concerning, patients can continue to have a partial response at 6 months and then have a repeat scan at 9 months. Patients can also develop metastatic disease at anytime during this period. This study classified patients as having complete response if this was achieved up to 6 months. Patients were classified as having treatment failure if they developed metastatic disease, had locoregional progression in the form of new positive lymph nodes or progression in size of the involved nodes or primary tumour, or if the MDT classified the patient as having residual disease up to the 6 months after completion of CRT.

2.2.3 Collection scheme

Figure 2.1 shows the sample collection scheme. Formalin-Fixed Paraffin-Embedded (FFPE) tissue taken as part of the diagnostic pathway was collected, and any tissue subsequently taken as part of clinical management – repeat biopsy at either the primary or possible secondary site, or APR for LRF- was also collected. Bloods were collected at six timepoints before, during, and after CRT. Timepoints were chosen for both pragmatic and scientific reasons. Pragmatically, timepoints correlate with when venepuncture is performed as part of their clinical care (before and during treatment as part of routine monitoring) or when patients are attending clinic and have corresponding response assessment scans (3 months and 6 months). Scientifically, the earlier timepoints during treatment are when we expect an acute immunological and inflammatory response. As discussed, a complete response is usually achieved 6 months after completion of CRT. The baseline, end of treatment, and after treatment time points are when we think IO could be started in patients at high risk of treatment failure. There is more recent evidence (published after this collection scheme was designed) suggesting that positive cHPV-DNA (defined as > 16 copies/ml) 3 months after CRT is the earliest timepoint that is prognostic for recurrence after treatment with CRT.

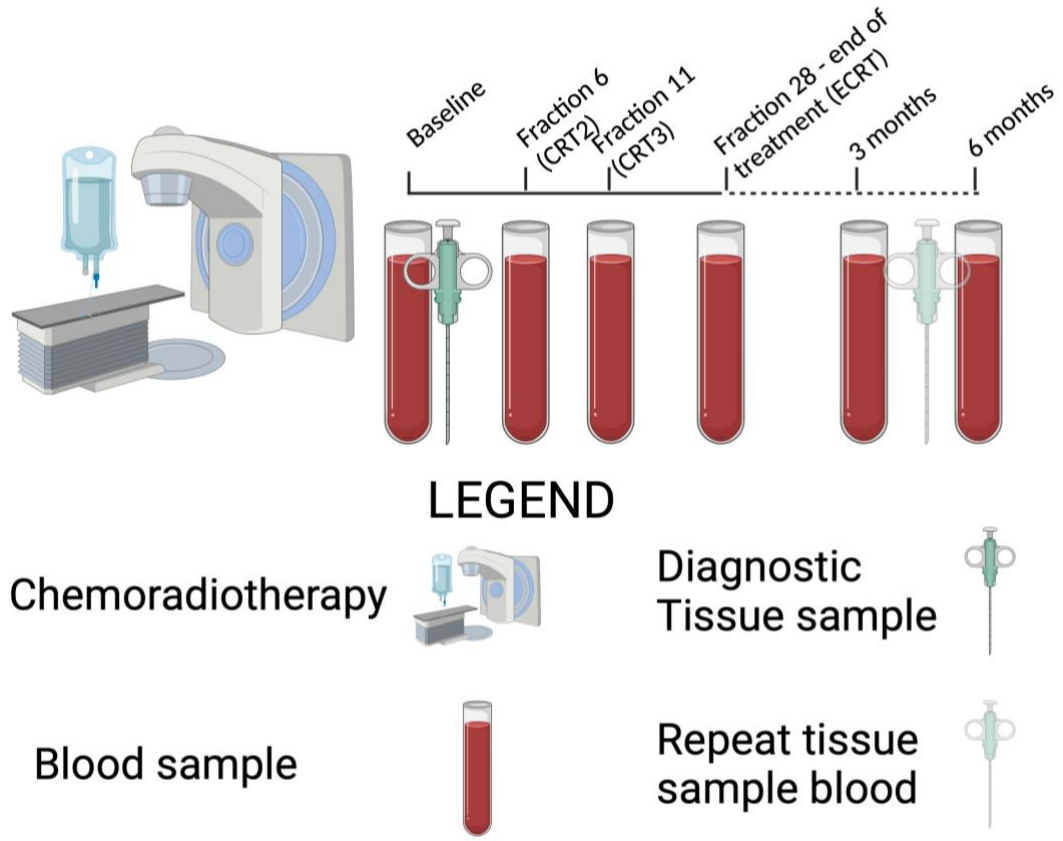


Figure 2.1 GRECIAN blood sample collection

2.2.4 Patient recruitment and sample compliance.

Across the three recruitment centres, 40 patients were recruited – 21 from Leeds, 10 from Manchester and 9 from Oxford. 8 patients had treatment failure at 6 months – 4 from Leeds and 4 from Manchester. Table 2.1 shows the blood sample collection. Of a possible 240 blood samples, 210 were collected, with one of these plasma only. Compliance was better in patients with complete response. Patients with treatment failure either went on to receive palliative chemotherapy, salvage surgery or best supportive care. They were not always on the same clinical pathway as those with a complete response, and as a result, 3 month and 6 month samples were often missed.

Table 2.1 GRECIAN blood sample collection compliance

Patient	Baseline	Week 2	Week 3	End of treatment	3 Months	6 Months
Leeds 1						
Leeds 2						
Leeds 3						
Leeds 4						

Patient	Baseline	Week 2	Week 3	End of treatment	3 Months	6 Months
Leeds 5						
Leeds 6						
Leeds 7						
Leeds 8						
Leeds 9						
Leeds 10						
Leeds 11						
Leeds 12						
Leeds 13						
Leeds 14						
Leeds 15						
Leeds 16						
Leeds 17						
Leeds 18						
Leeds 19						
Leeds 20						
Leeds 21						
Manchester 1						
Manchester 2						
Manchester 3						
Manchester 4						
Manchester 5						
Manchester 6						
Manchester 7						
Manchester 8						
Manchester 9						
Manchester 10						
Oxford 1				plasma only		

Patient	Baseline	Week 2	Week 3	End of treatment	3 Months	6 Months
Oxford 2						
Oxford 3						
Oxford 4						
Oxford 5						
Oxford 6						
Oxford 7						
Oxford 8						
Oxford 9						

2.3 Wet laboratory methods

2.3.1 Recipes and Reagents

Table 2.2 shows all recipes and reagents.

Table 2.2 Recipes and Reagents

Freezing media	90% Foetal calf serum (FCS, Scientific Laboratory Supplies) + *(v/v)* 10% dimethyl sulfoxide (DMSO)
Complete RPMI	Roswell Park Memorial Institute medium (RPMI, Sigma-Aldrich (SA)) + 10 % *(v/v)* FCS
PBS	Phosphate buffered saline tablet (PBS, SA) per 200ml de-ionised water
FACS buffer	PBS with 1 % (*v/v)* FCS and 0.1 % (*v/v)* sodium azide (SA)
Transcription factor Fixation/Permeabilization solution	25% (v/v) of Fixation/Permeabilization Concentrate with 75% (v/v) Fixation/Permeabilization Diluent
1 N Hydrochloric Acid	8.33% (v/v) of 12N HCl with 91.67% (v/v) of deionised water
1.2 N sodium hydroxide/0.5 M HEPES	12% (v/v) of 10 N NaOH and 88% (v/v) of deionised water. Add 11.9g of HEPES per 100ml of solution

2.3.2 Blood sample processing

Patient samples were ideally collected within 2 hours but could be up to 24 hours after venepuncture. Up to six 10ml EDTA blood sample tubes were collected per timepoint. Samples were centrifuged at 2000g for 10 minutes, with acceleration of 50%, and brake

at 30% to pellet cells. Separated plasma at the top of the EDTA tube was collected in 1ml aliquots and stored at -80°C for cytokine and cHPV-DNA assays. The middle white cell layer was mixed with 20ml of Hanks' Balanced Salt Solution (HBSS, SA) before 10ml was slowly added onto a Lymphoprep™ (Serumwurk Bernburg AG) layer in two 15ml Falcons and centrifuged at 800g for 20 minutes, acceleration 50%, brake 30%. The lymphocyte layer was transferred into a 50ml Falcon and washed twice with HBSS. Washes were done at 400g with 100% acceleration and brake for 5 minutes. The PBMCs were counted and then stored in 1ml aliquots at $1x10^6$ cells/ml in freezing media for flow cytometry. Cell counts were performed using Trypan Blue (Fisher Scientific) and a standard haemocytometer. Mr Frosty™ freezing containers were used to cool PBMCs to - 80 °C before being transferred to liquid nitrogen.

2.3.3 Flow cytometry

PBMCs were defrosted, washed, and a cell count was performed in complete RPMI. They were washed in PBS and then resuspended to 1x10^7 cells/ml in PBS. 100ul was added to a v-bottomed well on a 96-well plate. 100µl of one of six mastermixes was added and covered in foil for at least 30 minutes at room temperature. Table 2.3 shows the composition of each mastermix, and Table 2.4 gives details of each antibody. Apart from FOXP3, all antibodies are extracellular. Stained PBMCs were washed three times in 150µl of FACS buffer before being fixed in 150ul of PFA. Following surface marker staining, the regulatory T-cell wells were transferred to FACS tubes, and the eBioscience FOXp3/Transcription factor staining buffer set was used before acquisition. 1ml of Transcription factor Fixation/Permeabilization solution was added to each tube and covered in foil for 30 minutes. They were washed twice with 10 ml of permeabilisation buffer. 10ul of the FOXp3 antibody was added and covered in foil for 30 minutes. The wash was repeated twice before the PBMCs were resuspended in 150 PFA and added to the 96-well v-bottomed plate. The acquisition was performed on a Cytoflex LX (Beckman Coulter), and data was analysed using CytExpert software (Beckman Coulter). Distinct cell sub-populations and changes in markers in these sub-populations were measured using % positive expression. Fluorescence minus one (FMO) controls were made for each mastermix and used to set gates and support data interpretation. All washes are done at 400g with 100% acceleration and brake for 5 minutes.

Table 2.3 Mastermix composition

Mastermix	Targets
T-cells & Natural Killer cells #1	CD3, CD4, CD8, CD56, CCR7, PD-1, PD-L1, OX40, GITR, ICOS, CD69
T-cells & Natural Killer cells #2	CD3, CD4, CD8, CD56, CD25, CD137, TIGIT, CTLA-4, LAG-3, TIM-3, CD27, CD28

Mastermix	Targets
Regulatory T-cells	CD3, CD4, CD25, CD127, PD-1, PD-L1, FOXP3, TIGIT, CTLA-4, LAG-3, TIM-3, CD28, CD69, CD27
Monocytes and B-cells #1	CD14, CD16, PD-1, PD-L1, HLA-DR, CD86, CD80, ICOS-L, HVEM, CD40, CD69, CD19
Monocytes and B-cells #2	CD14, CD16, Gal-9, OX40L, HLA-ABC, CD137L, CD70, CD155, CD112, CD19
Dendritic cells	Lineage cocktail, CD123, PD-1, PD-L1, HLA-DR, CD86, CD80, CD1c, CD141, CD40, CD69, CD11c, CD83

Table 2.4 Antibody details

Target	Fluorophore	Clone	Supplier	Titrated volume per test (µl)
CD3	FITC	HIT3a	BD	1
CD4	APC-H7	RPA-T4	BD	1
PD-1 (CD279)	PE	MIH4	BD	20
PD-L1 (CD274)	PE-CF594	MIH1	BD	5
CD56	PE-Cy7	B159	BD	2
OX40 (CD134)	BV421	ACT35	BD	5
CCR7	BB700	3D12	BD	2
GITR	BV605	V27-580	BD	10
ICOS (CD278)	BV650	DX29	BD	2
CD40L (CD154)	BV750	TRAP-1	BD	2
BTLA (CD272)	BUV395	J168-540	BD	5
CD69	APC	FN50	BD	20
CD8	AF700	RPA-T8	BD	1
CD137	PE	4B4-1	BD	10
TIGIT	BV421	741182	BD	1
CD25	BB700	M-A251	BD	5
CTLA-4	BV605	BNI3	Biolegend	10
LAG-3	BV650	11C3C65	Biolegend	2

Target	Fluorophore	Clone	Supplier	Titrated volume per test (µl)
TIM-3 (CD366)	BV786	7D3	BD	2
CD28	BUV395	CD28.2	BD	1
CD27	APC	MT271	BD	5
CD127	APC-R700	HIL-7R-M2	BD	2
FoxP3	PE-Cy7	FJK-16s	ThermoFisher	10
CD14	FITC	M5E2	BD	5
CD16	BB700	3-G8	BD	1
CD86	BV421	BU63	BD	1
HLA-DR	PE-Cy7	G46-6	BD	2
CD80	BV605	L307.4	BD	2
ICOS-L (CD275)	BV650	2D3/B7-H2	BD	1
HVEM (CD270)	BV786	CW10	BD	5
CD40	BUV395	5C3	BD	2
CD19	APC-H7	SJ25C1	BD	1
GAL-9	PE-Cy7	9M1-3	Biolegend	5
OX40-L (CD252)	BV421	Ik-1	BD	5
HLA-ABC	BV605	G46-2.6	BD	5
CD137-L	BV650	C65-485	BD	5
CD70	BV786	Ki-24	BD	2
CD155	BUV395	SKII.4	BD	1
CD112	APC	TX31	Biolegend	1
Lineage cocktail	FITC		BD	5
CD123	BB700	7G3	BD	1
CD1c	BV650	F10/21A3	BD	1
CD141	BV786	1A4	BD	1

Target	Fluorophore	Clone	Supplier	Titrated volume per test (µl)
CD11c	AF700	B-ly6	BD	1
CD83	APC-Cy7	HB15e	Biolegend	1
Viability stain	BV510		BD	10

2.3.4 Multiplex cytokine – human screening panel

The human screening panel used the Bio-Plex Pro Human Cytokine Assay (Bio-Rad #12007283) kit. Details of the reagents used can be found in the instruction manual. Plasma samples were defrosted and centrifuged at room temperature, and the particulate debris was discarded. Plasma was diluted 1:4 in sample diluent. A fourfold standard dilution series was prepared using reconstituted standard and standard diluent. Magnetic beads were diluted 10:1 in Assay Buffer. 50ul of magnetic bead solution was added to the flat-bottomed 96-well plate supplied and washed twice in wash buffer using the Bio-Plex handheld magnetic washer. 50ul of samples, standards, blanks, and controls are added to each corresponding well. The plate was covered and incubated at 850 rpm for 30 minutes at room temperature. The plate was washed three times, and 25ul of detection antibodies were added to each well. The plate was covered and incubated at 850 rpm for 30 minutes at room temperature. 50µl of streptavidin was added to each well. It was incubated at 850 rpm for 10 minutes at room temperature. The plate was washed three times, resuspended in 125µl assay buffer, and read on a Bioplex 2200 reader (BioRad). Table 2.5 shows the cytokines measured in this panel.

Table 2.5 Human Cytokine Screening Panel

β -Nerve Growth Factor (β-NGF)	Cutaneous T-cell Attracting Chemokine (CTACK)	Eosinophil Chemotactic Protein (Eotaxin)
Fibroblast Growth Factor, Basic (FGF basic)	Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	Granulocyte Colony-Stimulating Factor (G-CSF)
Growth-Regulated Oncogene α (GRO-α)	Hepatocyte Growth Factor (HGF)	Interferon Alpha 2 (IFN-α2)
Interferon-γ (IFN-γ)	Interleukin-1α (IL-1α)	Interleukin-1β (IL-1β)
Interleukin 1 Receptor Antagonist (IL-1ra)	Interleukin 2 (IL-2)	Interleukin 2-Receptor α (IL-2Rα)
Interleukin 3 (IL-3)	Interleukin 4 (IL-4)	Interleukin 5 (IL-5)
Interleukin 6 (IL-6)	Interleukin 7 (IL-7)	Interleukin 8 (IL-8)

Interleukin 9 (IL-9)	Interleukin 10 (IL-10)	Interleukin 12 Subunit Alpha (40 kDa) (IL-12 (p40))
Interleukin 12 (70 kDa) (IL-12 (p70))	Interleukin 13 (IL-13)	Interleukin 15 (IL-15)
Interleukin 16 (IL-16)	Interleukin 17A (IL-17A)	Interleukin 18 (IL-18)
Interferon γ -Inducible Protein 10 (IP-10)	Leukaemia Inhibitory Factor (LIF)	Monocyte Chemoattractant Protein-1 (MCP-1)
Monocyte Chemoattractant Protein-3 (MCP-3)	Macrophage Colony-Stimulating Factor (M-CSF)	Monokine Induced by γ Interferon (MIG)
Macrophage Inflammatory Protein 1 Alpha (MIP-1α)	Macrophage Inflammatory Protein 1 Beta (MIP-1β)	Macrophage Migration Inhibitory Factor (MIF)
Platelet-Derived Growth Factor-BB (PDGF-BB)	Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES)	Stem Cell Factor (SCF)
Stem Cell Growth Factor-β (SCGF-β)	Stromal Cell-Derived Factor 1α (SDF-1α)	Tumour Necrosis Factor-α (TNF-α)
Tumour Necrosis Factor-β (TNF-β)	TNF-Related Apoptosis-Inducing Ligand (TRAIL)	Vascular Endothelial Growth Factor (VEGF)

2.3.5 Multiplex cytokine – TGF-β

The Bio-Plex Pro TGF-β 3-plex assay (Bio-Rad #171W4001M) was used to measure the three different isoforms of TGF-β in plasma. The accompanying instruction manual details the reagents used. Plasma samples were defrosted and centrifuged at room temperature, and the particulate debris was discarded. To measure immunoreactive TGF-β, the plasma must be activated and then neutralised. To activate, 5µl of 1 N HCl was added to 25µl of plasma, mixed thoroughly and left for 10 minutes at room temperature. To neutralise, 5µl of 1.2 N NaOH/0.5 M HEPES was added to the sample. 365µl of sample diluent was added to the sample, totalling 400µl. A fourfold standard dilution series was prepared using reconstituted standard and standard diluent. Magnetic beads were diluted 20:1 in Assay Buffer. 50µl of magnetic bead solution was added to the flat-bottomed 96-well plate supplied and washed twice in wash buffer using the Bio-Plex handheld magnetic washer. 50µl of activated samples, standards, blanks, and controls were added to each corresponding well. The plate was covered and incubated at 850 rpm for 2 hours minutes at room temperature. The plate was then washed three times with 100µl of wash buffer. 25µl of detection antibody was added, and the plate was covered and incubated at 850 rpm for 1 hour at room temperature. The plate was then washed three times with 100µl of wash buffer. 50µl of

streptavidin -PE was added to each well. The plate is covered and incubated at 850 rpm for 30 minutes at room temperature. The plate is washed three times, resuspended in 125µl assay buffer, and read on a Bioplex 2200 reader (BioRad).

2.3.6 Data Visualisation and Statistics

GraphPad Prism was used for data visualisation and statistical tests. Choice and justification for statistical tests are discussed within each chapter.

2.4 RT-IO ASCC Systematic review methodology

2.4.1 Review question

What are the trial designs, treatment regimens and translational endpoints for RT-IO combination trials in ASCC?

2.4.2 Search Strategy

The systematic review was prospectively registered with PROSPERO(CRD42023384068). Interventional clinical trials (Phase I to IV inclusive) of metastatic or localised ASCC, or trials including other tumour types where separate analysis of > 5 anal cancer patients was possible, that combined RT with treatments to modulate or stimulate the immune system were included. Non-interventional trials, trials where a study protocol has been registered but the study withdrawn or never occurred, or trials investigating the immunomodulatory effects of the current standard of care treatment, where the principal recognised mechanism of action of this treatment is not immunomodulatory, were excluded. Trial registries ClinicalTrials.gov and EudraCT were searched on 14 August 2023. Initial search terms included MeSH and synonyms of anal cancer AND immunotherapy. This resulted in known trials being excluded from the search terms. Therefore, search terms were reduced to anal cancer and its associated terms/MeSH headings where search engines allowed. PubMed was searched using the keywords “anal cancer” AND “immunotherapy,” and results were filtered for clinical trials only. Trials that met the inclusion criteria from trial registry databases were searched on PubMed for more detailed and up-to-date publications. Appendix 1 shows the search strategy and inclusion/exclusion criteria.

2.4.3 Data extraction and quality assessment

A standardised data extraction sheet was used to record information from the included trials. Data was extracted from trial protocols, publications, and trial registration websites. Data extraction was enhanced by undertaking a grey literature search for the trial protocol when it was not published or available on the registry website. If it could not be found, the trial’s corresponding author or principal investigator was emailed requesting it, recognising that this may have limited success due to commercial sensitivities. Extracted data included cancer type, stage of ASCC disease, IO

intervention and mechanism of action, IO regimen in relation to CRT, chemotherapy regimen, RT regimen, geographical location, Phase of trial, number of experimental arms, randomisation status, biomarker selection, (Proposed) sample size, trials status (not yet recruiting, recruiting, active not recruiting, published), protocol availability, statistical design, primary outcome(s), secondary outcome(s), and translational/exploratory analysis performed.

2.4.4 Quality assessment and statistical analysis

The objectives of this review were not the efficacy or outcomes of RT-IO ASCC trials. Given the authors' prior knowledge of this area, most of the trials included were expected to have not yet published any results, and those that had would be from small sample sizes. Meta-analysis of a small number of trials with small sample sizes increases the risk of bias and, therefore, was not performed[174]. Quality assessment tools such as RoB2 and ROBIN-I use outcomes and effect size as part of their scoring and do not cover areas such as translational analysis plans, treatment regimens, or patient selection in sufficient detail for the objectives of this review. Therefore, no formal quality assessment tools were used. Formal measures of heterogeneity, such as I^2, quantify the percentage of the total variation across studies due to heterogeneity rather than chance. However, these measures for statistical heterogeneity based on effect sizes are not used for clinical or methodological heterogeneity and were not performed [175].

2.5 CORMAC-2 Methodology

2.5.1 Study overview

CORMAC-2 was conducted through a three-step process, initially identifying existing definitions through systematic review, using these to populate a two-round Delphi questionnaire (completed by 51 experts from 13 countries), and finally, ratification through an online consensus meeting. The study protocol was published online a priori [176].

An international steering committee was established to ensure the validity of the Delphi questionnaire content and promote broader global awareness and participation. Members comprised oncologists, colorectal surgeons, and clinical trialists with leading roles in past and current clinical trials in CRT for ASCC. E-mail invitations to senior authors of published and active trials of CRT for ASCC formed the steering committee.

2.5.2 Patient and public involvement

A group of patient and public representatives were recruited through the Leeds Radiotherapy Research Group Public and Patient Involvement (PPI) group (UK) and the Anal Cancer Foundation (USA). The academic research language used to describe nuanced differences in survival and disease activity outcome definitions was

considered by the steering committee and the PPI group to be too technical to allow meaningful participation of patients and the public in the Delphi questionnaire and consensus meeting. However, consideration of patients' views was considered important, especially where outcome definition or measurement may involve burdensome or invasive investigations. PPI groups were therefore asked about the impact and acceptability of different modalities and frequencies of outcome assessment, and their feedback was summarised and presented to participants during the Delphi questionnaire.

2.5.3 Selection of outcomes

CORMAC-2 focused on outcomes in the disease activity and survival domains, which require a standardised definition. Outcomes in the toxicity and life impact domains require a different approach, involving identifying and recommending suitable measurement instruments as described by the COSMIN guidelines [177]. This represents substantial work involving a methodology different from that employed here and is beyond the scope of this phase of the project.

From the survival domain, two outcomes were excluded. Firstly, OS was consistently and unambiguously defined, and therefore, the steering committee agreed that there was no benefit to including it in voting in the Delphi questionnaire or consensus meeting. Secondly, the identified definitions of PFS and DFS were found to have significant overlap. After extensive discussion amongst the steering committee, it was decided that due to this overlap, only one would be included and that disease-free survival was the more applicable term in the context of non-metastatic, curative intent trials (the scope of the CORMAC-COS). A summary of the steering committee discussion on DFS versus PFS and the rationale for the decision can be available from the authors on request.

2.5.4 Systematic review update

The CORMAC systematic review was updated to 11th February 2021. Details of the systematic review, including search strategy, eligibility, and exclusion criteria, can be found on PROSPERO (CRD42016036540). See Appendix 2 for the PRISMA flow diagram. Definitions for the 11 disease activity and survival outcomes in the CORMAC-COS were identified and extracted verbatim. Results from the systematic review update were presented to the steering committee to facilitate accurate summarisation of existing definitions into Delphi question items.

2.5.5 Delphi questionnaire

2.5.5.1 Recruitment

Healthcare professionals were eligible to participate in the Delphi questionnaire if they have been involved in the design, recruitment, running or publication of anal cancer

research and trials. The CORMAC-2 Delphi questionnaire was promoted at the International Multidisciplinary Anal Cancer Conference (IMACC) 2021 through the National Cancer Institute (NCI) Rectal-Anal Taskforce (USA) and active trial networks such as PLATO (UK), IMACC (International) and subcommittees of the National Cancer Research Institute (UK) and NRG Oncology (North America). Steering committee members used their knowledge of local societies, meetings, email lists and contacts to increase participation in the questionnaire. Potential participants could register their interest via the CORMAC website before the study opened [178].

2.5.5.2 Questionnaire

Delphi question items were constructed from the outcome definitions identified in the systematic review. Disease activity outcomes were broken down to cover aspects of timing and modality of assessment and grading/assessment criteria. Composite outcomes (e.g. disease-free survival) were separated to rate the inclusion of all potential events, as previously described by the Definition for the Assessment of Time-to-event Endpoints in CANcer trials (DATECAN) group [179]. The Delphi questionnaire was run on the online DelphiManager platform [180]. Consent to participate was obtained from participants at registration along with demographic information including their discipline, country of practice and role in ASCC research. During each of the 2 rounds, for each outcome, participants were asked to rate the importance of adopting a particular definition on a Likert scale of 1 (limited importance) to 9 (critical importance). Instructions for completing the questionnaire were included at the start of each round, and links to information necessary for answering questions were provided, for example, details of RECIST criteria [181]. A summary of the relevant PPI feedback was provided alongside each definition. Participants had the opportunity to provide free text feedback on each question and to suggest alternative definitions (figure 1). In round 2, participants were shown the summarised results from round 1, including their own round 1 score for each item, the summarised scores from other participants (as a histogram), and relevant summarised feedback from the free text responses (anonymised). They were then asked to consider this information before re-scoring each item.

2.5.5.3 Consensus criteria

Criteria for consensus were agreed a priori and published in the study protocol. All items from Round 1 were retained for Round 2. After the final round, each definition option was assigned to one of three categories:

1. Consensus in: 70% or more respondents rate the item as critically important (7–9) AND 15% or fewer rate the item as limited importance (1–3). Unless an issue is raised at the consensus meeting, it is included in the final definitions.

2. Consensus out: 50% or less of respondents rate the item as critically important (7-9). Unless an issue is raised at the consensus meeting, it is excluded in the final definitions.

3. No consensus

2.5.6 Consensus meeting

All participants completing both rounds of the Delphi questionnaire were invited to participate in the online consensus meeting along with the steering committee members. All participants who registered to participate in the consensus meeting were sent a summary of the Delphi questionnaire results before the meeting. The meeting was chaired by an independent clinician researcher who was not part of the steering committee, had expertise in COS methodology, and in chairing similar meetings (SM). The definitions that met "consensus in" and "consensus out" criteria after the final round of the Delphi questionnaire were presented but were not voted on again unless consensus meeting participants raised a fundamental problem with that definition. "No consensus" definitions were shown, and group discussion was facilitated. The chair ensured different views were heard and all participants could voice their opinions. Following this, anonymous voting was conducted using the same 9-point Likert scale and consensus criteria used in the Delphi questionnaire. If no consensus was found on the first vote, further discussion and a second vote was performed. Anonymous online voting was conducted using Mentimeter software [182].

2.5.7 Registration and ethics

The protocol was prospectively registered on protocolexchange[176]. As per the University of Manchester ethics decision tool, no ethical approval was required as it was a study soliciting professional opinions, all personal information collected was publicly available and participants agreed for their details to be shared as part of collaborative authorship [183].

CORMAC is registered with the COMET initiative [184].

Chapter 3 Cellular Markers of immune resistance to chemoradiotherapy for locally advanced anal cancer

3.1 Introduction

This chapter aims to evaluate the cellular markers of immune resistance from standard-of-care CRT for locally advanced ASCC. Around 30 % of patients with locally advanced disease experience treatment relapse after CRT, with the majority of these occurring in the first 6 months after treatment [71].

Immunophenotyping by flow cytometry of patient PBMCs allows for the identification of distinct populations such as T-cells, B-cells and DCs. Based on the expression of specific markers, cells within these populations can be functionally classified - for example, CD4+ T-cells, CD8+ Cytotoxic T-cells and FOXP3+CD25^{high}CD127^{low} regulatory T-cells. Individual co-inhibitory or co-stimulatory proteins can be identified on these functionally classified cell types. These can represent specific resistance markers that could be targeted in future clinical trials. Dynamic changes observed through serial sampling will enhance our understanding of these markers in response to CRT and will aid in determining the optimal timing for RT-IO combinations.

3.2 Patient samples available for analysis

Although 40 patients were recruited to GRECIAN due to operator error on the cytoflex machine, only 38 patients were available for flow cytometry analysis. Not all the 210 samples with PBMCs collected had enough viable PBMCs on defrosting to allow complete immunophenotyping. Two possible reasons were insufficient blood volumes or cell degradation due to slow processing. These both occurred stochastically throughout sample collection and were not related to specific time points or recruitment centres. CRT causes lymphopenia that worsens as treatment continues. As a result, samples at CRT2, CRT3 and ECRT timepoints were more likely to have insufficient PBMCs for full immunophenotyping analysis. Given that ICIs are the only approved type of IO for combination with RT, Treg and T &NK tubes were prioritised over the B-cell & monocyte and DC tubes if there was inadequate PBMCs for full analysis.

Patients who had early treatment failure may go on to receive early salvage surgery or palliative chemotherapy prior to completing the translational blood sample scheme. Therefore, fewer 3M and 6M timepoints were available for analysis in treatment failure compared to patients with complete response.

Occasionally, specific markers may have expression dramatically different from other comparable samples. If expression was particularly low, this may have occurred due to the omission of an antibody from the mastermix. If there was a particularly small population of cells within that sample, expression could be extreme in either direction

(for example if there were low CD8+ cells in a sample, the expression of PD-1 may be much higher or lower than that of a comparable sample with a larger population). Such samples were removed from the analysis.

3.3 Statistical methods - considerations

The following factors were considered in decisions on the choice of statistical test and appropriate adjustments for multiple comparisons within this chapter.

1. As discussed in the introduction, immune cell type and the markers expressed on these immune cells are of interest and are analysed separately.
2. From a statistical point of view, the type of immune cells and the markers they express were split into targeted and exploratory. Targeted markers have a stronger scientific rationale to justify their evaluation. They have a reasonable body of evidence in other tumours or any evidence that they may be important in ASCC. Targeted cell types are CD8+, CD4+, Regulatory T-cells (Tregs), NK cells and DCs. Targeted markers are PD-1, PD-L1, CTLA-4, and TIGIT. All other cell types and markers are exploratory, with limited evidence in other tumour types, or have never been reported in ASCC. Statistical adjustment was performed separately for targeted and exploratory markers and cell types.
3. This chapter aims to discover immune resistance markers. Adjusted statistical testing to control for false discovery is essential to identify the most promising markers to take forward into potential future clinical trials.
4. This data has never been explored in locally advanced ASCC patients receiving CRT. In the future, these results may be confirmed in a different cohort, evaluated using other techniques such as mass spectroscopy, or measured in diagnostic primary tissue using immunohistochemistry. At this stage of research into ASCC immune resistance markers, we want to ensure sufficiently high power to correctly reject null hypotheses when they are true. Therefore, methods of adjustment that are less conservative and have a higher power for discovery were used.
5. Expression of a specific marker on a particular immune cell type is likely related to the same marker on a different immune cell type. For example, PD-1 expression on a CD8+ T cell is likely associated with PD-1 expression on a CD4+ T cell within the same sample. These tests are not entirely independent and thus multiple testing between cells may result in an inflated type I error.
6. Markers on the same biological pathway collectively contribute to a biological effect. These may be on different cell types - such as Ox40 and Ox40-ligand – or even on the same cell type - CD28 and CTLA-4 on CD4+ cells. Interpreting these results in a broader context rather than as isolated tests is important.
7. It is important to consider whether markers identified may be prognostic of outcome or predictive of the response to CRT. Therefore, not only is this project identifying immune resistance markers at baseline, but also how dynamic

changes in these markers may help to suggest the best timing to integrate IO with CRT.

8. Figure 3.1 shows hypothetical differences that may be observed, and which may point toward either neoadjuvant, concurrent or adjuvant IO. Statistical tests performed need to be able to identify these different patterns of response. Therefore, it is not only differences between complete response and treatment failure groups that are important, but changes within each group in response to treatment.

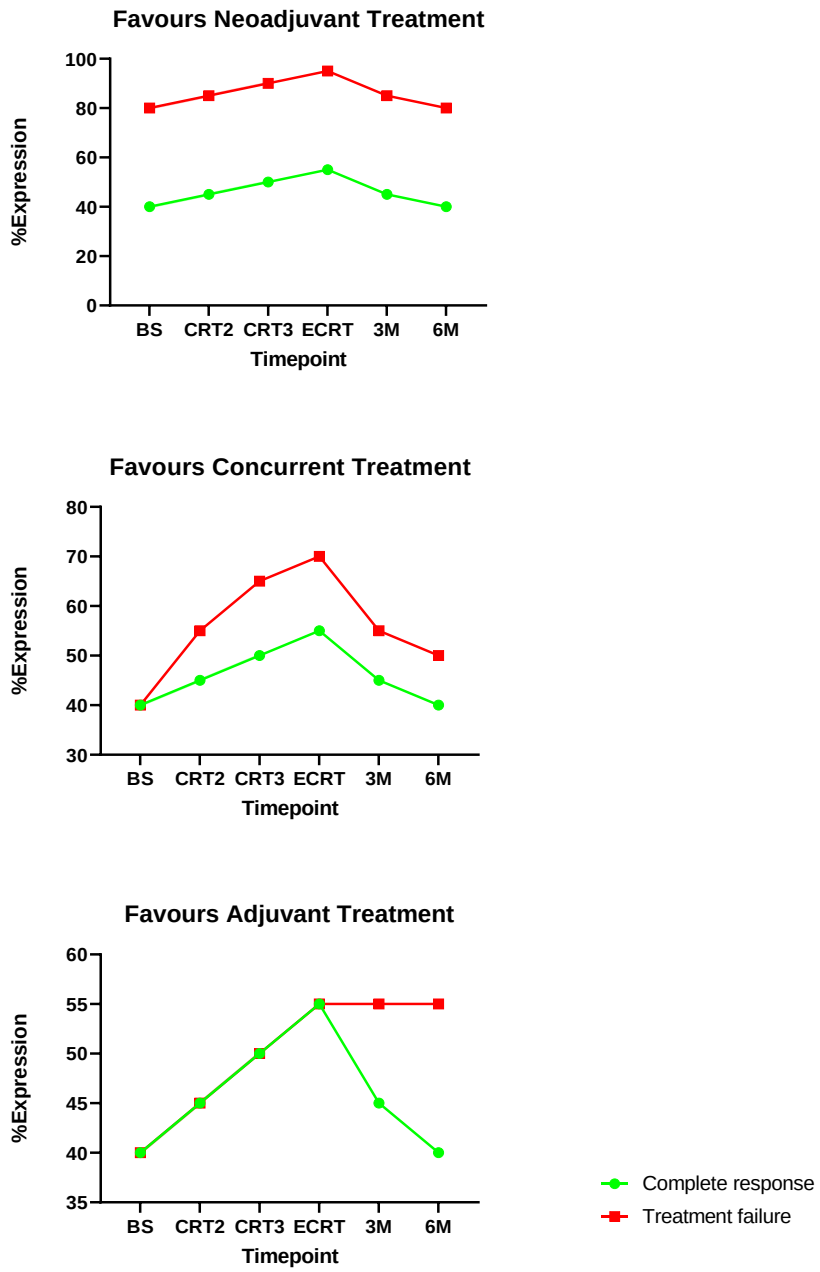


Figure 3.1 Theoretical dynamic changes in a co-inhibitory molecule that would favour neoadjuvant, concurrent or adjuvant treatment, respectively.

3.4 Statistical analysis plan

The data produced by flow cytometry in this context is expressed as % expression (dependent quantitative variable) separated according to response outcome (independent categorical variable) and timepoint (independent categorical variable). Figure 2.1 from the methods chapter shows the timepoints for blood sample collection. Given the chapter's aims, the type of data available and the statistical considerations described, the following statistical comparisons were performed.

1) Baseline comparisons between groups (between complete response versus treatment failure)
2) ECRT comparisons between groups
3) 6M comparisons between groups.
4) Comparison within groups, comparing BS versus ECRT (for example, baseline complete response versus ECRT complete response)
5) Comparison within groups, comparing ECRT versus 6M

These comparisons allow for the optimal timing of IO and dynamic changes within groups to be assessed, while controlling the number of unnecessary statistical comparisons. Data distribution was measured at baseline for each marker/cell type. Student t-tests were used for normally distributed data and Mann-Whitney U tests were used for skewed data. Sensitivity analysis comparing test results, data transformation and removal of outliers were used to aid this decision. For comparisons within groups and comparisons between groups, corrections for multiple testing using a 2-step Benjamini-Hochberg procedure to control the False Discovery Rate (FDR) was used to account for dependency between tests. The significance level for the false discovery rate was set at 0.05. The q-value for each test in the Benjamini-Hochberg procedure is the adjusted p-value. The p-values reported on each graph represent adjusted p-values. The Benjamini-Hochberg table is shown for the first set of adjustments to show how p-values are calculated. Regardless of the results of the above statistical tests, trends in the data that relate to possible immune resistance markers results deemed to have biological relevance are discussed. Figure 3.2 shows the grouping for statistical adjustment. Dynamic changes for all markers and cell populations are presented. Individual comparisons for targeted markers, and individual comparisons for all significant exploratory results following FDR adjustment are shown.

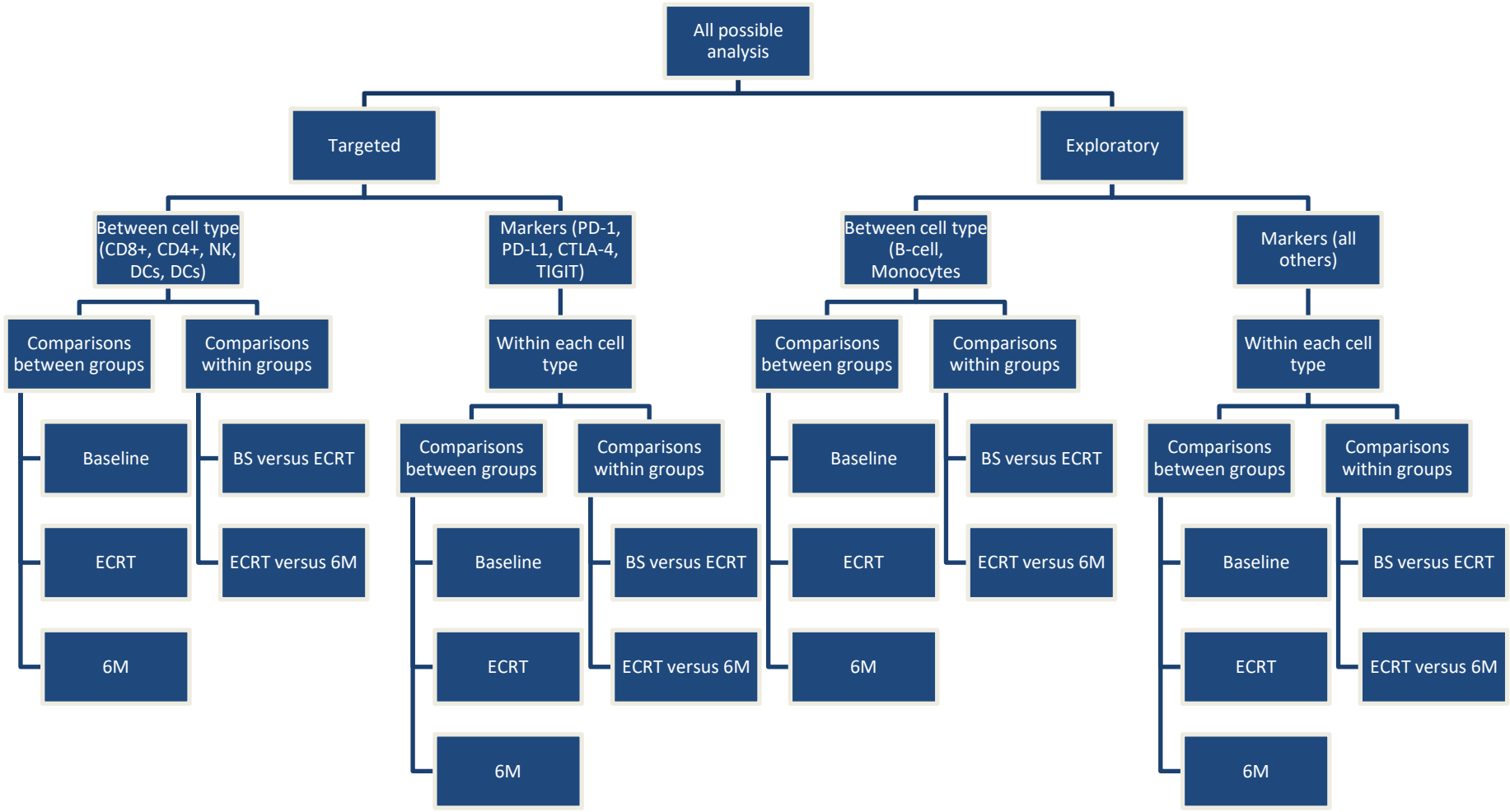


Figure 3.2 Split for statistical analysis and adjustment

3.5 Targeted Immune cell populations

Figure 3.3 shows dynamic changes in Treg, CD8+ T-cell, CD4+ T-cell and NK cell populations for treatment failure and complete response. Figure 3.4 and Figure 3.5 show comparisons between treatment failure and complete response (between groups) at baseline, ECRT and 6M for Treg, CD8+ T-cell, CD4+ T-cell and NK cell populations respectively. As shown in Figure 3.4, there is a statistically higher Treg population in patients with treatment failure compared to those with complete response at baseline (44.74 % Tregs in treatment failure at baseline versus 20.73 % in complete response, adjusted p-value 0.0025). Although not statistically significant, there is a trend for a higher Treg population in treatment failure to be maintained at ECRT (57.43 % Tregs in treatment failure at ECRT versus 31.43 % in complete response, adjusted p-value 0.077) and 6M (42.62 % Tregs in treatment failure at 6M versus 24.5 % in complete response, adjusted p-value 0.234). There were no differences between complete response and treatment failure for the CD8+ T-cells, CD4+ T-cells or NK cells populations.

Figure 3.6 and Figure 3.7 show comparisons within groups for BS to ECRT and ECRT to 6m for Treg, CD8+ T-cells, CD4+ T-cells and NK cell populations respectively. In complete response there was a trend for an increase in mean Treg population from BS to ECRT (20.73 % Tregs at BS in complete response to 31.42 % at ECRT, adjusted p-value 0.091) with a trend towards a decrease in mean Treg population from ECRT to 6M (31.42 % Tregs at ECRT in complete response to 24.58 % at 6M, adjusted p-value 0.497). A similar trend was seen in the treatment failure group with an increase from BS to ECRT (44.74 % Tregs at BS in treatment failure to 57.43 % at ECRT, adjusted p-value 0.715), and a decrease from ECRT to 6M (57.43 % Tregs at ECRT in treatment failure to 42.62 % at 6M, adjusted p-value 0.747). There was minimal change in CD8+ T-cells in response to treatment in either the treatment failure or complete response groups.

For CD4+ T-cells, there was a trend towards a decrease from BS to ECRT in complete response (48.43% CD4+ T-cells at BS in complete response to 38.87% at ECRT, adjusted p-value 0.071), with minimal change from ECRT to 6M. There was minimal change in CD4+ T-cells in response to treatment in treatment failure. There was a statistically significant increase from ECRT to 6M in the complete response group for NK cells (14.13 % NK cells at ECRT in complete response to 24.84 % at 6M, adjusted p-value 0.0039), with a similar trend seen in the treatment failure group (15.36 % NK cells at ECRT in treatment failure to 22.71 % at 6M, adjusted p-value 0.716). To demonstrate how these adjusted p-values are calculated Table 3.1 and Table 3.2 shows the result of multiple t-tests for between and within comparisons respectively. After the first 6 patients were analysed, it was clear that there was an insufficient population of DCs to adequately compare complete response and treatment failure. Given the cost of running each sample, and the low numbers of PBMCs, the DC panel was dropped from final analysis.

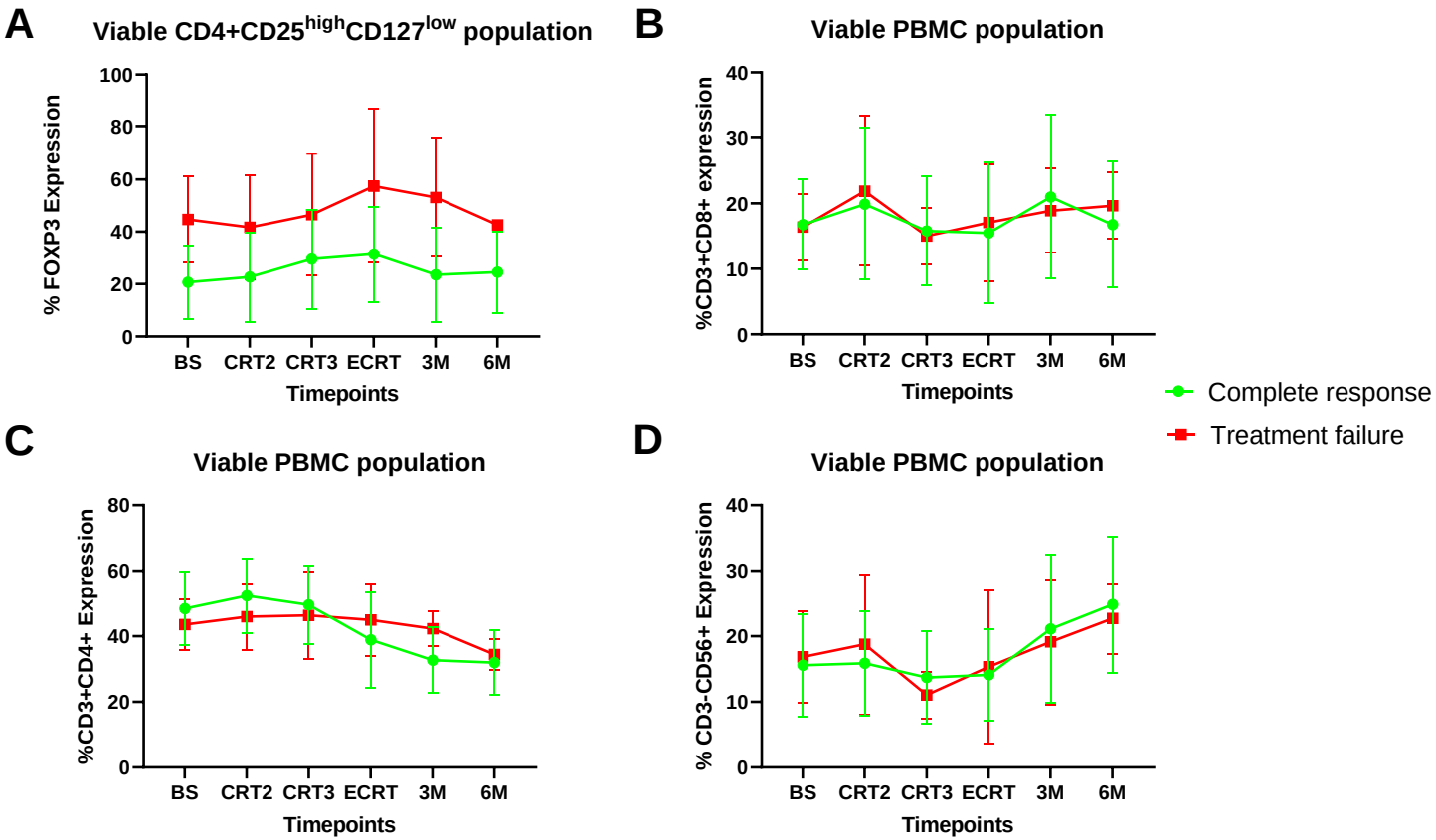


Figure 3.3 Dynamic changes in cell populations for A) Tregs B) CD8+ T-cells C) CD4+ T-Cells D) NK cells. For Tregs, data is presented as % positive of CD4+CD25^{high}CD127^{low} gate +/-SD. For CD8+ T-cells, CD4+ T-Cells and NK cells, data is expressed as % positive of viable PBMC gate +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

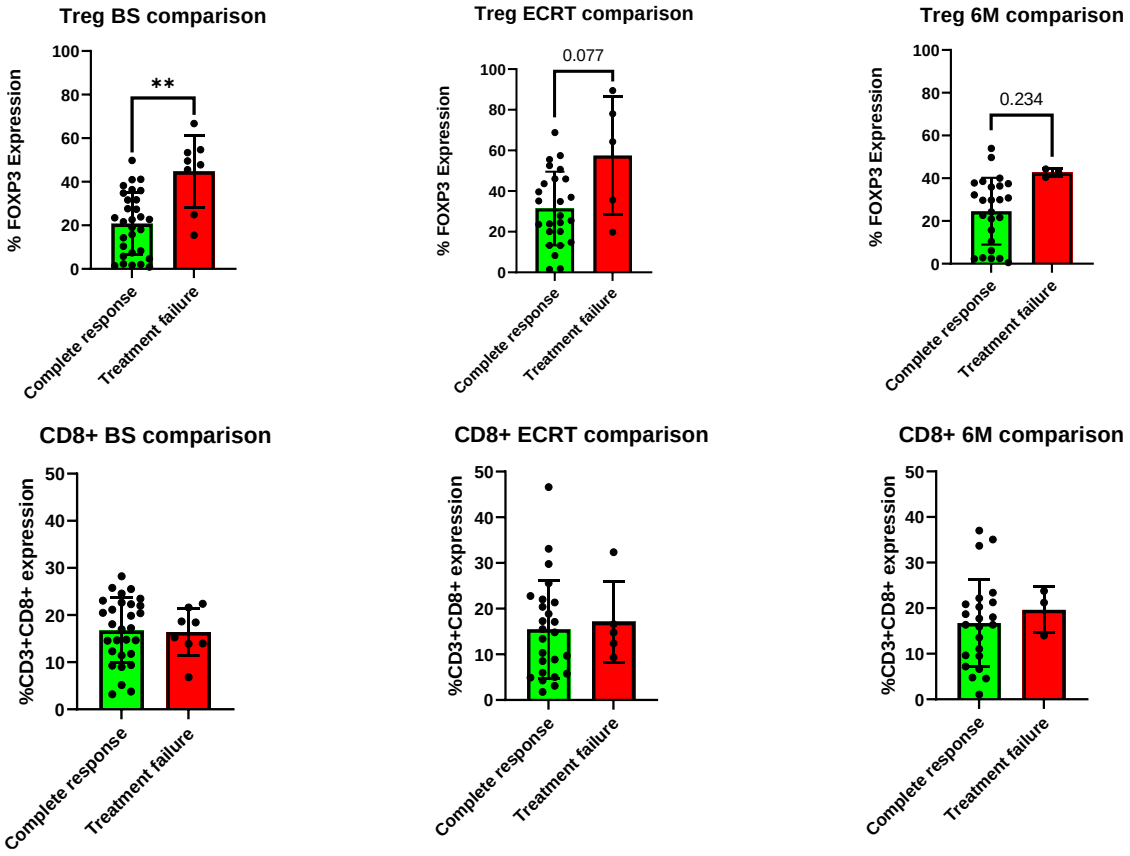


Figure 3.4 Comparison between complete response and treatment failure groups for Tregs and CD8+ T-cells at baseline, end of treatment and 6 months. For Tregs, data is presented as % positive of CD4+CD25^{high}CD127^{low} gate +/-SD. For CD8+ T-cells, data is expressed as % positive of viable PBMC gate +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱✱P ≤ 0.01

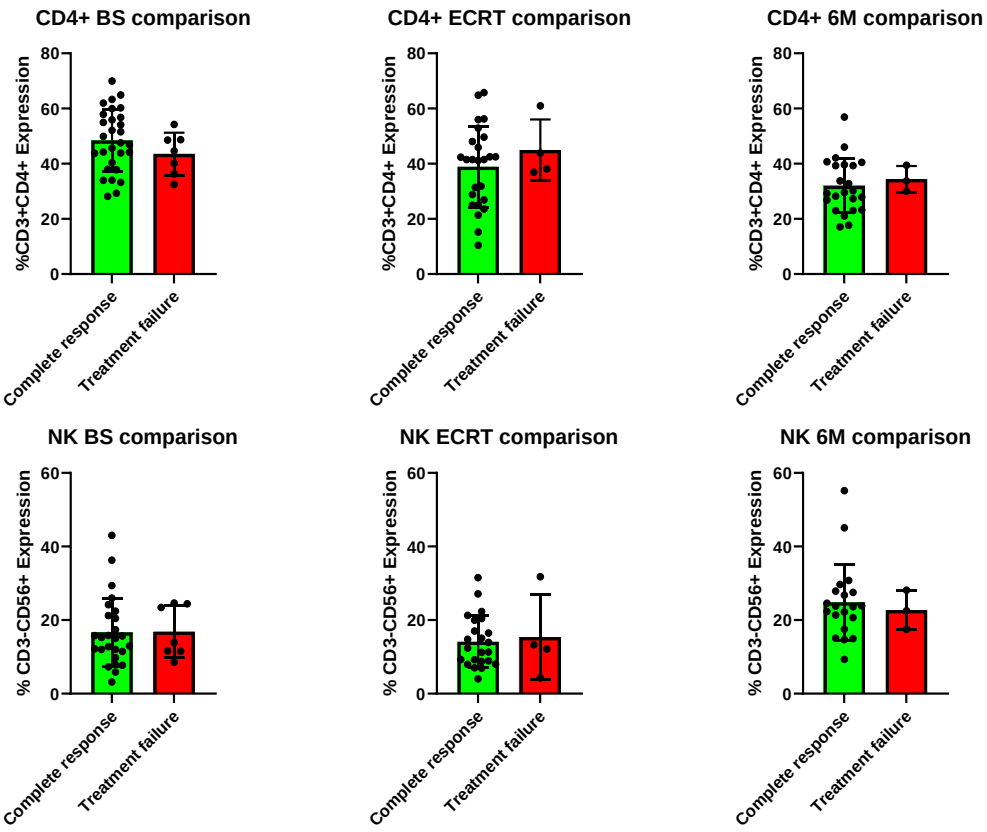


Figure 3.5 Comparison between complete response and treatment failure groups for CD4+ T-cells and Natural Killer cells at baseline, end of treatment and 6 months. Data is expressed as % positive of viable PBMC gate. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

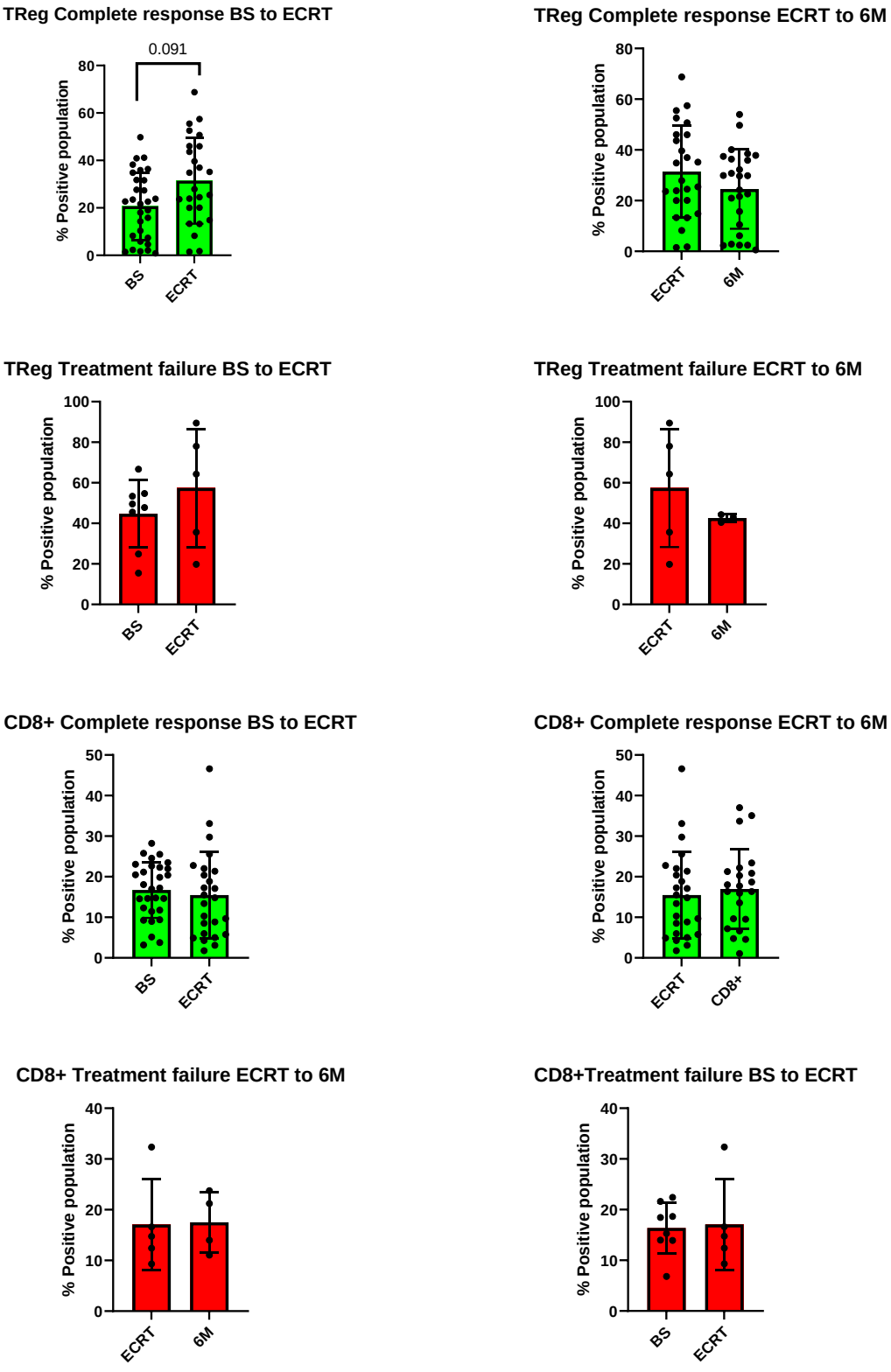


Figure 3.6 Changes in Tregs and CD8+ T-cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. For Tregs, data is presented as % positive of CD4+CD25^{high}CD127^{low} gate +/-SD. For CD8+ T-cells, data is expressed as % positive of viable PBMC gate +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

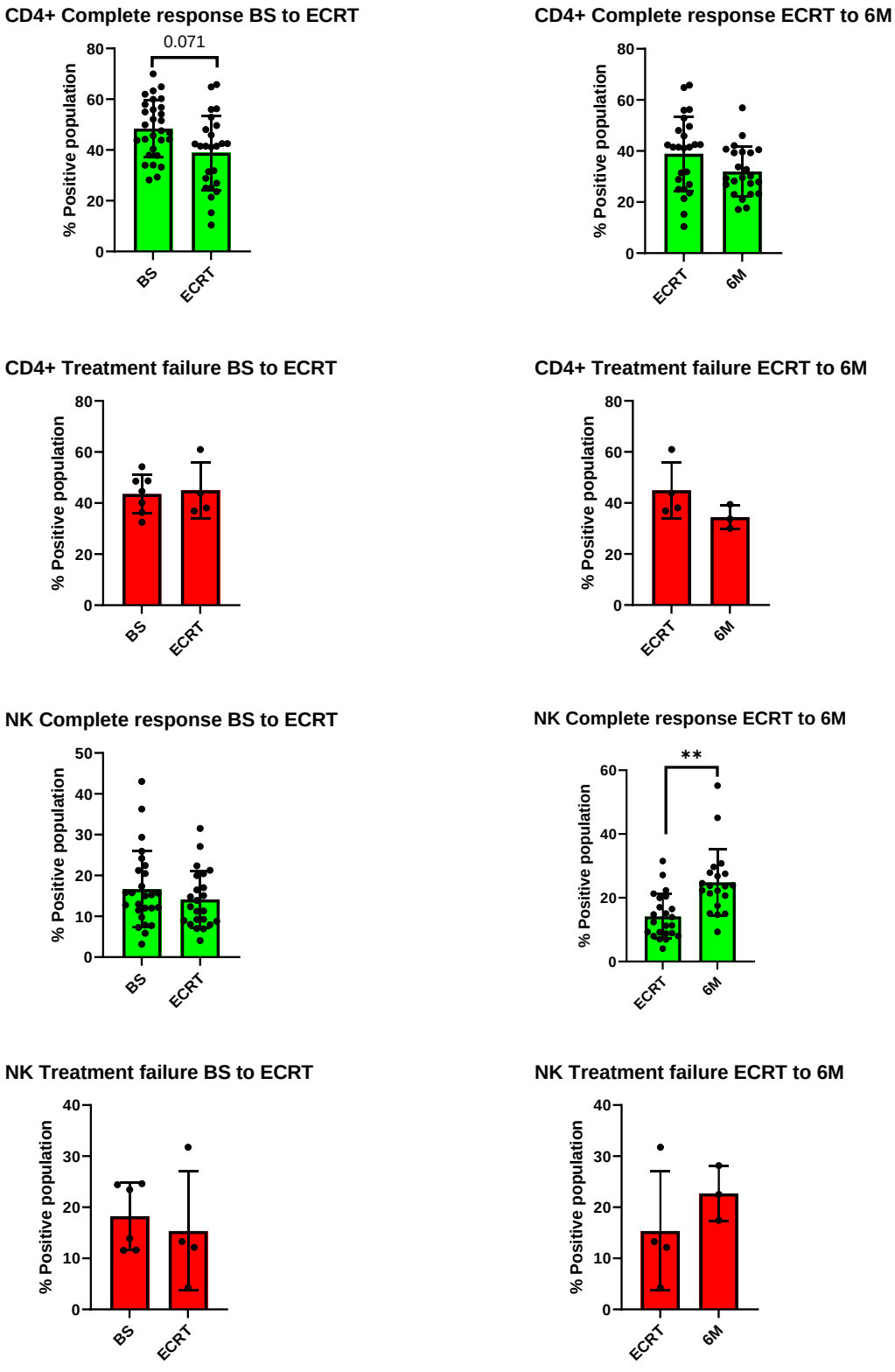


Figure 3.7 Changes in CD4+ T-cells and Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of viable PBMC gate +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

Table 3.1 Benjamini-Hochberg table for multiple t-tests between groups in targeted immune cell populations. Discovery refers to an adjusted p-value <0.05

Test	Discovery?	P value	q value (adjusted p-value)
TREG			
BS	Yes	0.0002	0.0025
ECRT	No	0.0133	0.0765
6M	No	0.0607	0.2338
CD8+			
BS	No	0.8928	0.8594
ECRT	No	0.8311	0.8594
6M	No	0.6144	0.8594
CD4+			
BS	No	0.2890	0.8344
ECRT	No	0.4358	0.8594
6M	No	0.6795	0.8594
NK			
BS	No	0.4005	0.8388
ECRT	No	0.7713	0.8594
6M	No	0.7344	0.8594

Table 3.2 Benjamini-Hochberg table for multiple t-tests within groups in targeted immune cell populations

Test	Discovery?	p-value	q-value (adjusted p-value)
TREG			
Complete response BS to ECRT	No	0.0174	0.0914
Complete response ECRT to 6M	No	0.1600	0.4967
Treatment failure BS to ECRT	No	0.3335	0.6362
Treatment failure ECRT to 6M	No	0.4269	0.6724
CD8+			
Complete response BS to ECRT	No	0.6016	0.7562
Complete response ECRT to 6M	No	0.6159	0.7562
Treatment failure BS to ECRT	No	0.8582	0.9012
Treatment failure ECRT to 6M	No	0.9388	0.9241
CD4+			
Complete response BS to ECRT	No	0.0090	0.0710
Complete response ECRT to 6M	No	0.0636	0.2505
Treatment failure BS to ECRT	No	0.8124	0.9012
Treatment failure ECRT to 6M	No	0.1892	0.4967
NK			
Complete response BS to ECRT	No	0.2950	0.6362
Complete response ECRT to 6M	Yes	0.0003	0.0039
Treatment failure BS to ECRT	No	0.6242	0.7562
Treatment failure ECRT to 6M	No	0.3636	0.6362

3.6 Exploratory Immune cell populations

Figure 3.8 shows dynamic changes in monocytes and B-cell populations for complete response and treatment failure. No statistical differences between groups were found. Figure 3.9 shows the comparison within groups. There was no change from BS to ECRT for monocytes in complete response or treatment failure groups. There was a statistically significant decrease in mean monocytes population from ECRT to 6M in complete response (76.86 % monocytes at ECRT in complete response to 69.6 % at 6M, adjusted p-value 0.006) and treatment failure (81.3% monocytes at ECRT in treatment

failure to 70.1 % at 6M, adjusted p-value 0.006). There was a statistically significant decrease in B-cells from BS to ECRT for complete response (11.85 % B-cells at BS to 7.26 % at ECRT, adjusted p-value 0.0023) and treatment failure (16.12 % B-cells at BS in treatment failure to 5.82 % at ECRT, adjusted p-value 0.0023). This was followed by a statistically significant increase from ECRT to 6M for complete response (7.26 % B-cells at ECRT in complete response to 12.84 % at 6M, adjusted p-value 0.0099) and treatment failure (5.82 % B-cells at ECRT in treatment failure to 18.74 % at 6M, adjusted p-value 0.0007).

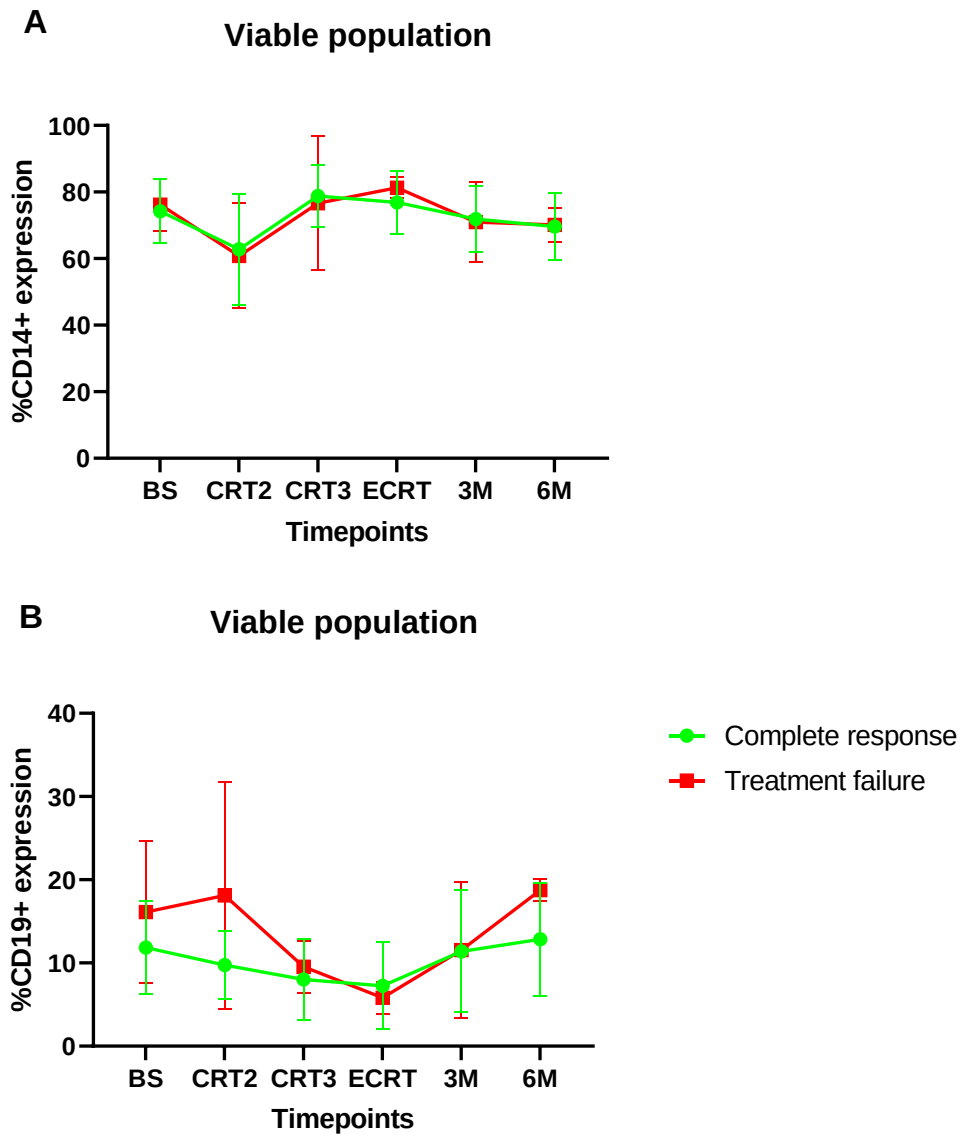


Figure 3.8 Dynamic changes in cell populations for A) Monocytes B) B-cells. Data is expressed as % positive of viable population gate +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

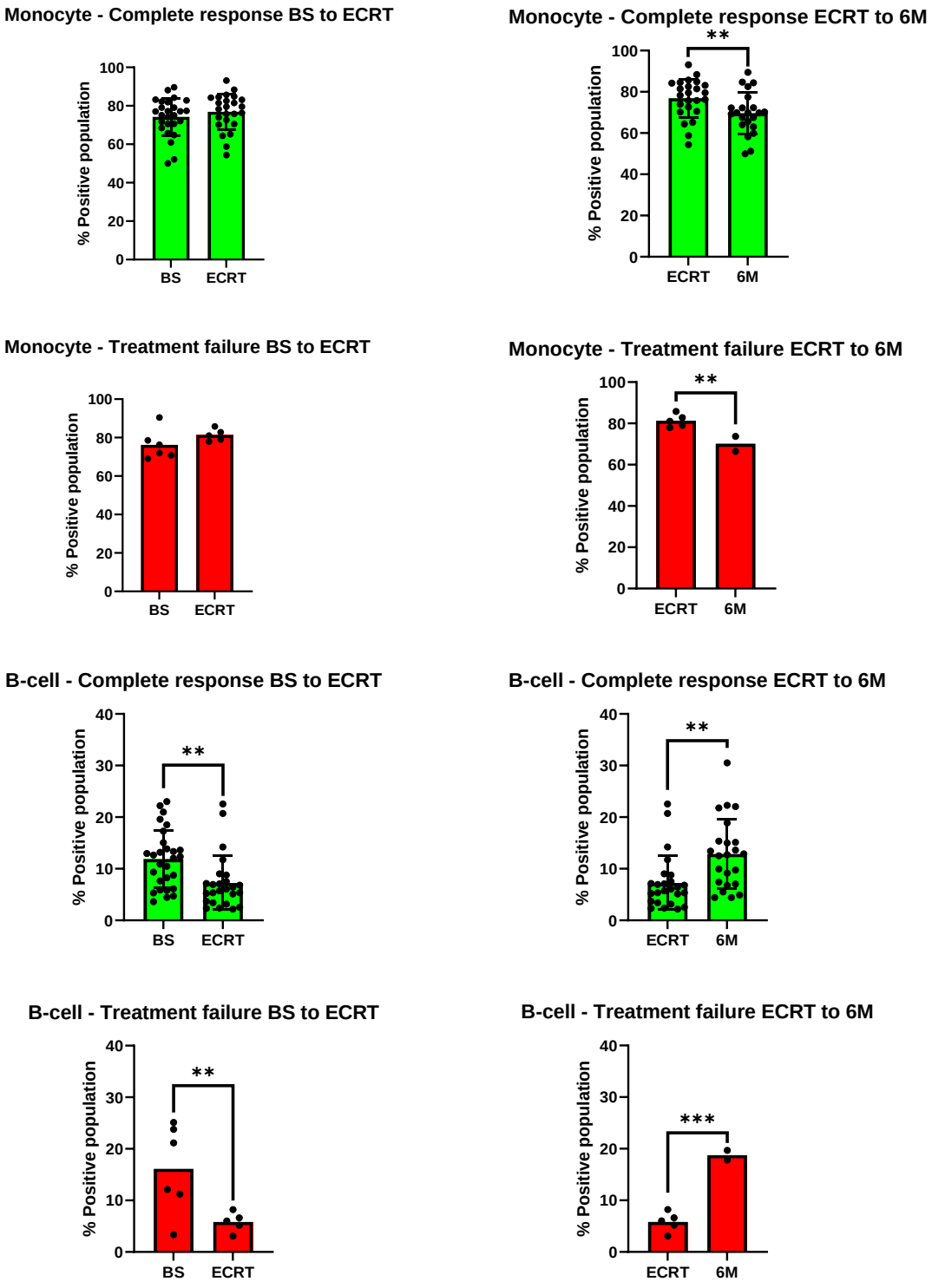


Figure 3.9 Changes in monocytes and B-cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of viable population gate +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱✱P ≤ 0.01, ✱✱✱P ≤ 0.001

3.7 Regulatory T-cells – targeted markers

Tregs were classified as FOXP3+CD4+CD25^{high}CD127^{low}. Figure 3.10 compares the expression of various co-inhibitory markers between FOXP3+CD4+CD25^{high}CD127^{low} and CD4+FOXP3- at baseline. This shows higher expression of co-inhibitory proteins TIGIT, CTLA-4, PD-1, LAG3 and TIM-3 in the FOXP3+ group, demonstrating that this classification has an immune suppressive phenotype.

Figure 3.11 shows dynamic changes in TIGIT, CTLA-4, PD-1 and PD-L1 expression on Tregs for complete response and treatment failure. Figure 3.12 and Figure 3.13 compare TIGIT, CTLA-4, PD-1 and PD-L1 expression on Tregs at BS, ECRT and 6M for complete response and treatment failure, respectively. As shown in Figure 3.12, there was a statistically significant higher expression of CTLA-4 on Tregs at baseline, ECRT and 6M timepoints in treatment failure compared to complete response (15.6 % CTLA-4 expression at baseline in treatment failure versus 5.9 % in complete response, adjusted p-value 0.015), (33.3 % CTLA-4 expression at ECRT in treatment failure versus 11.6 % in complete response, adjusted p-value 0.042), (43.4 % CTLA-4 expression at 6M in treatment failure versus 8.3 % in complete response, adjusted p-value 0.014). As shown in Figure 3.13, there was a trend for higher TIGIT at baseline in the treatment failure group (92.5 % TIGIT expression at baseline in treatment failure versus 86.3 % in complete response, adjusted p-value 0.225), but this difference was not maintained at ECRT or 6M.

Figure 3.14 and Figure 3.15 show changes during and after treatment for these markers within groups. Within groups, there were no statistically significant differences.

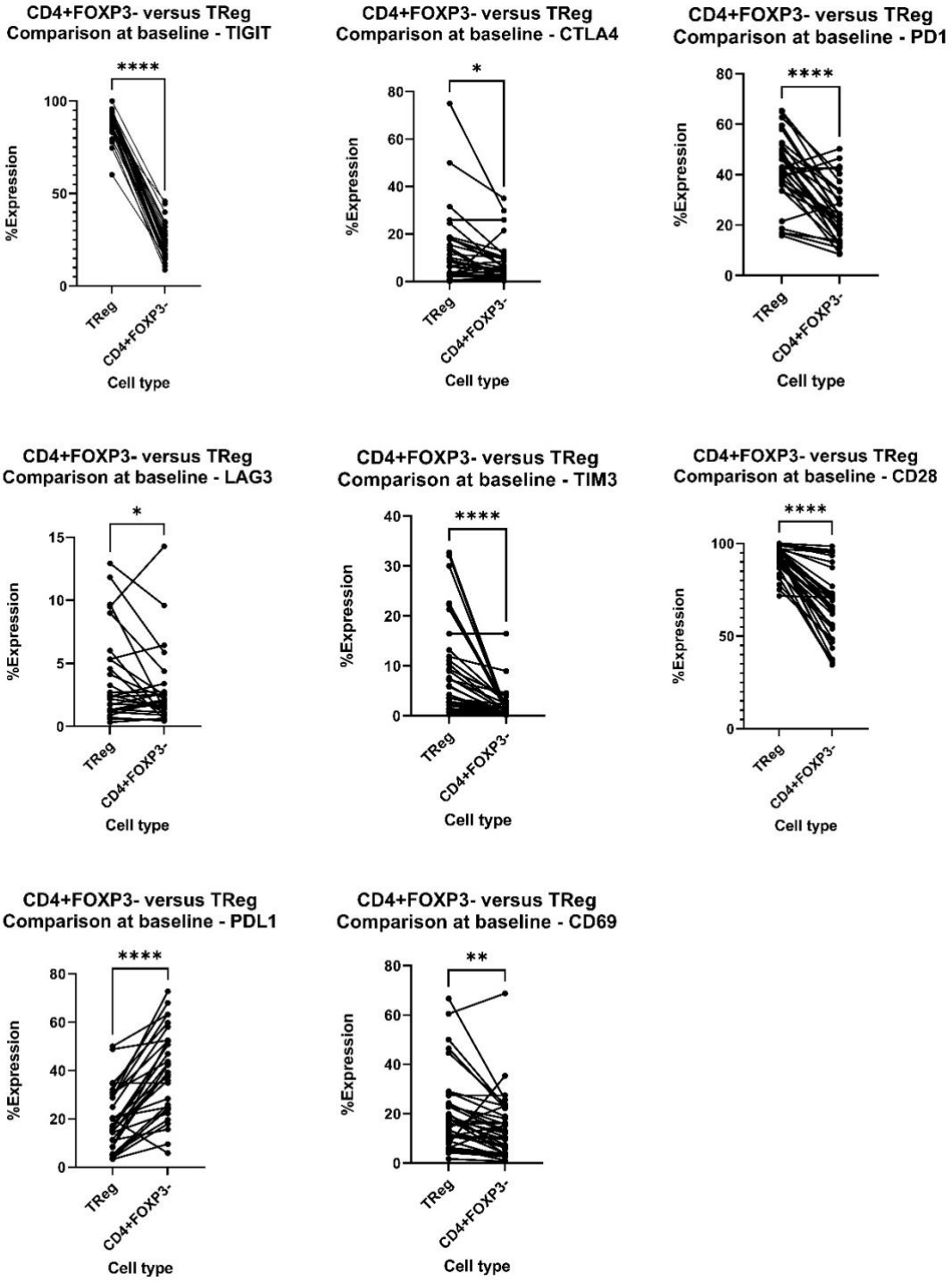


Figure 3.10 Expression of immune proteins at baseline on CD4+FOXP3- versus Treg (CD4+FOXP3+CD25highCD127low). Data is expressed % Expression for each marker, with resulted paired from each population within baseline samples. N=38 (Complete response and treatment failure combined)

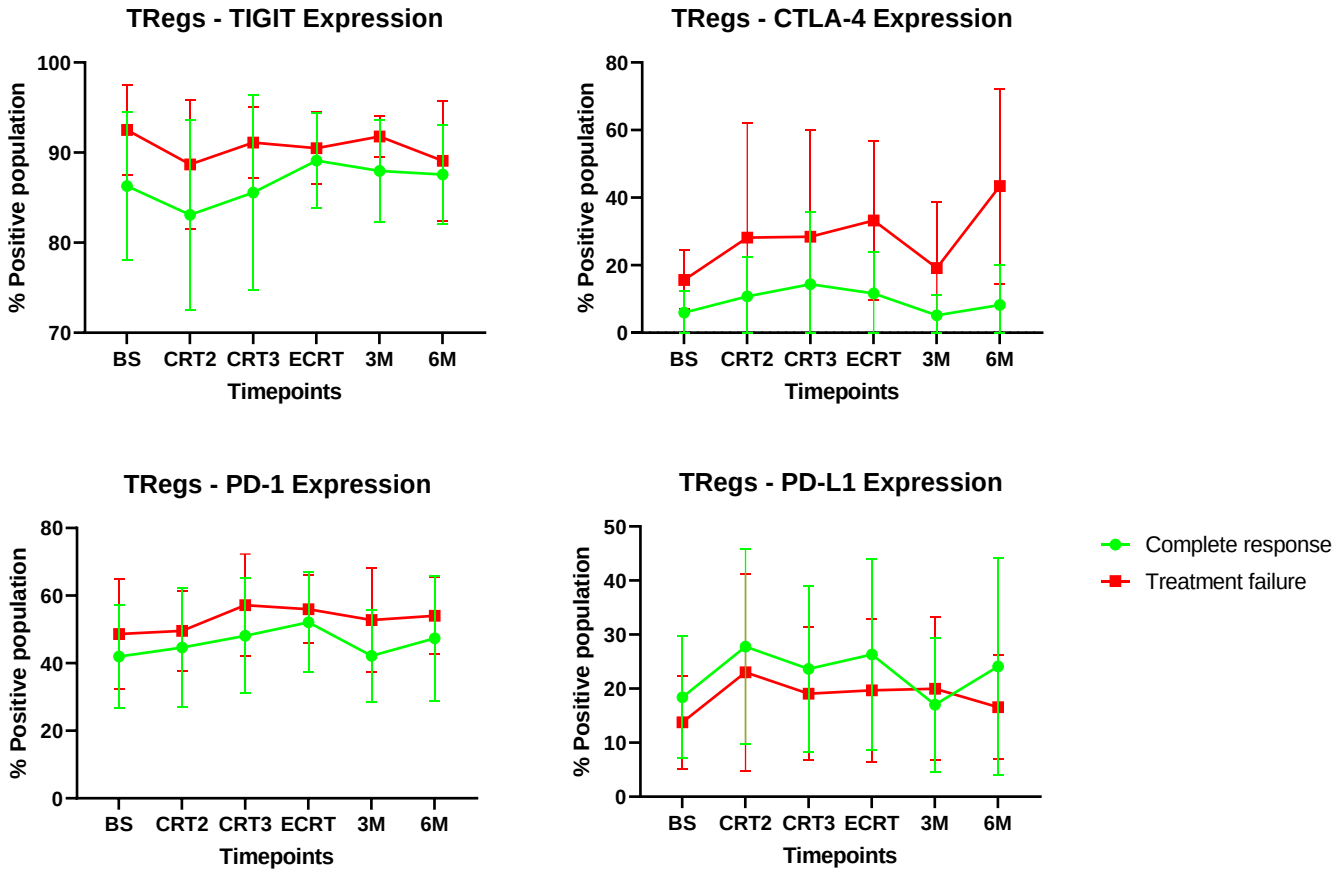


Figure 3.11 Dynamic changes in expression of TIGIT, CTLA-4, PD-1 and PD-L1 on Tregs. Data is expressed as % positive of Treg population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

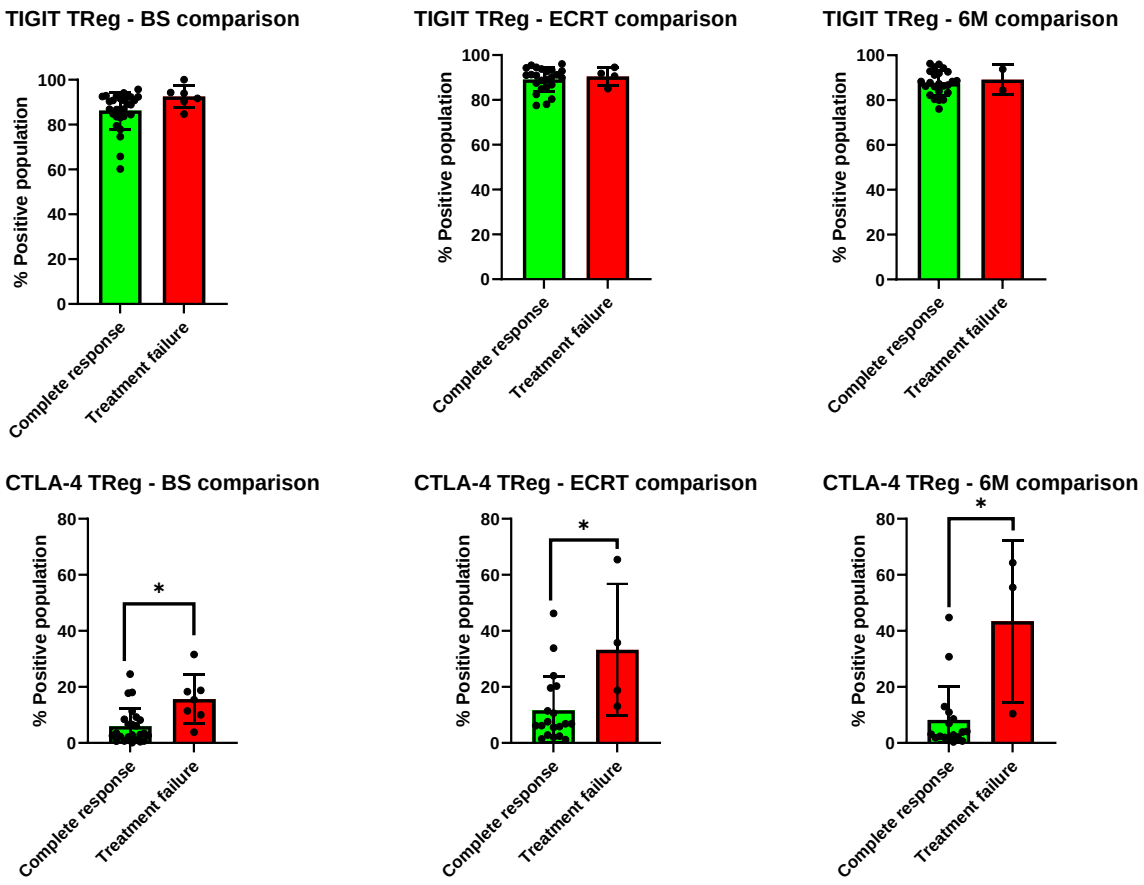


Figure 3.12 Comparison between complete response and treatment failure groups for TIGIT and CTLA-4 on Tregs. Data is expressed as % positive of Treg population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05

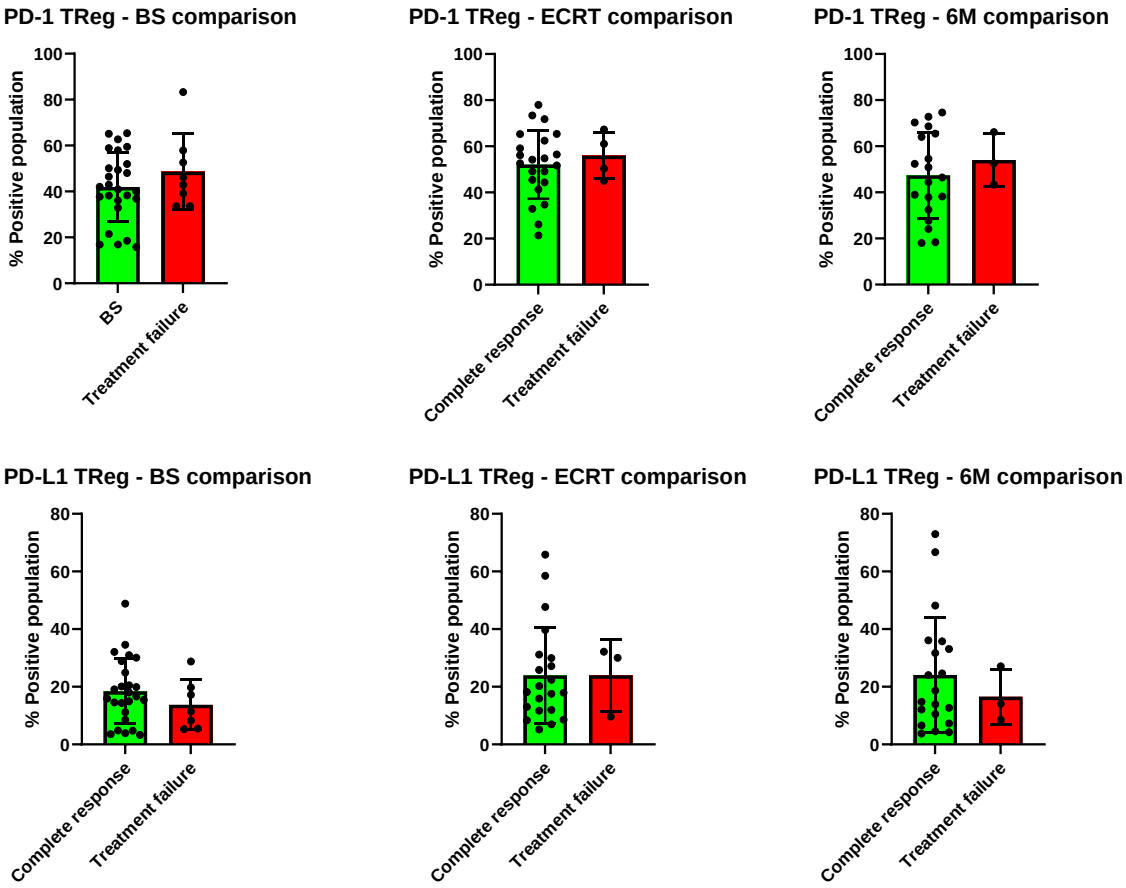


Figure 3.13 Comparison between complete response and treatment failure groups for PD-1 and PD-L1 on Tregs. Data is expressed as % positive of Treg population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

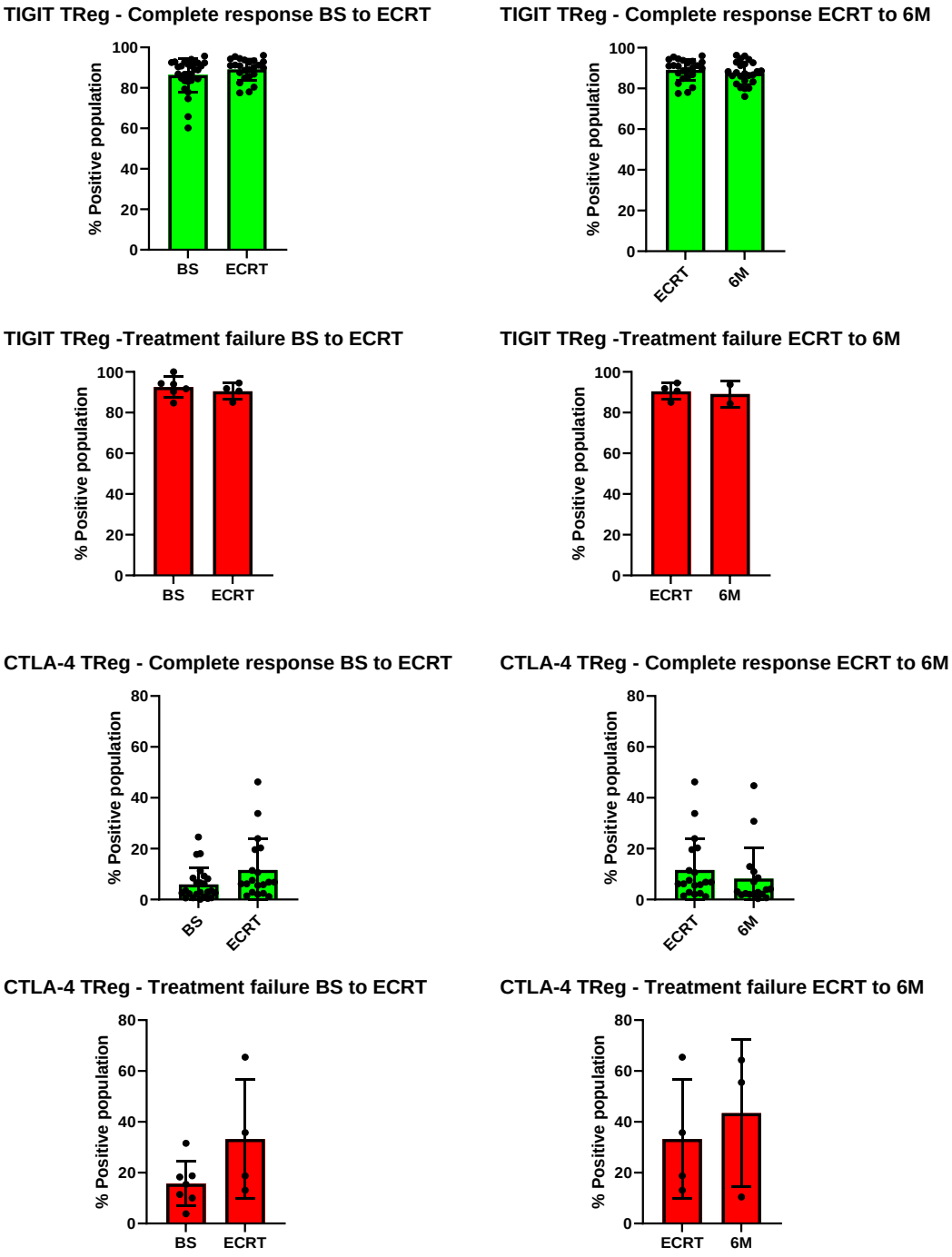


Figure 3.14 Changes in TIGIT and CTLA-4 Expression on Tregs from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Treg population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

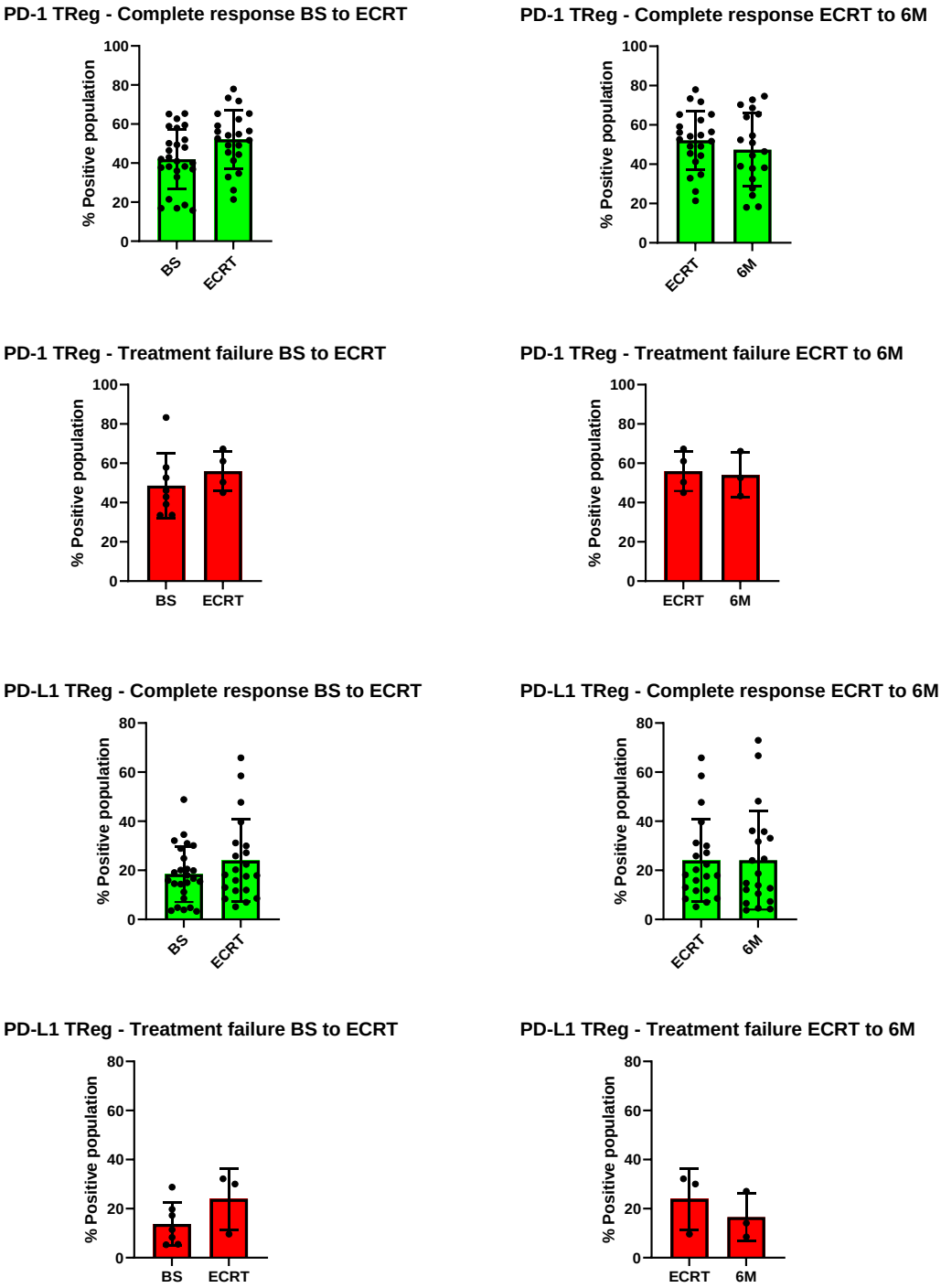


Figure 3.15 Changes in PD-1 and PD-L1 Expression on Tregs from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Treg population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

3.8 Regulatory T-cells – exploratory markers

Figure 3.16 shows dynamic changes in LAG-3, TIM-3, CD28 and CD69 Expression in treatment failure and complete response groups. No statistically significant results were seen between groups or within groups.

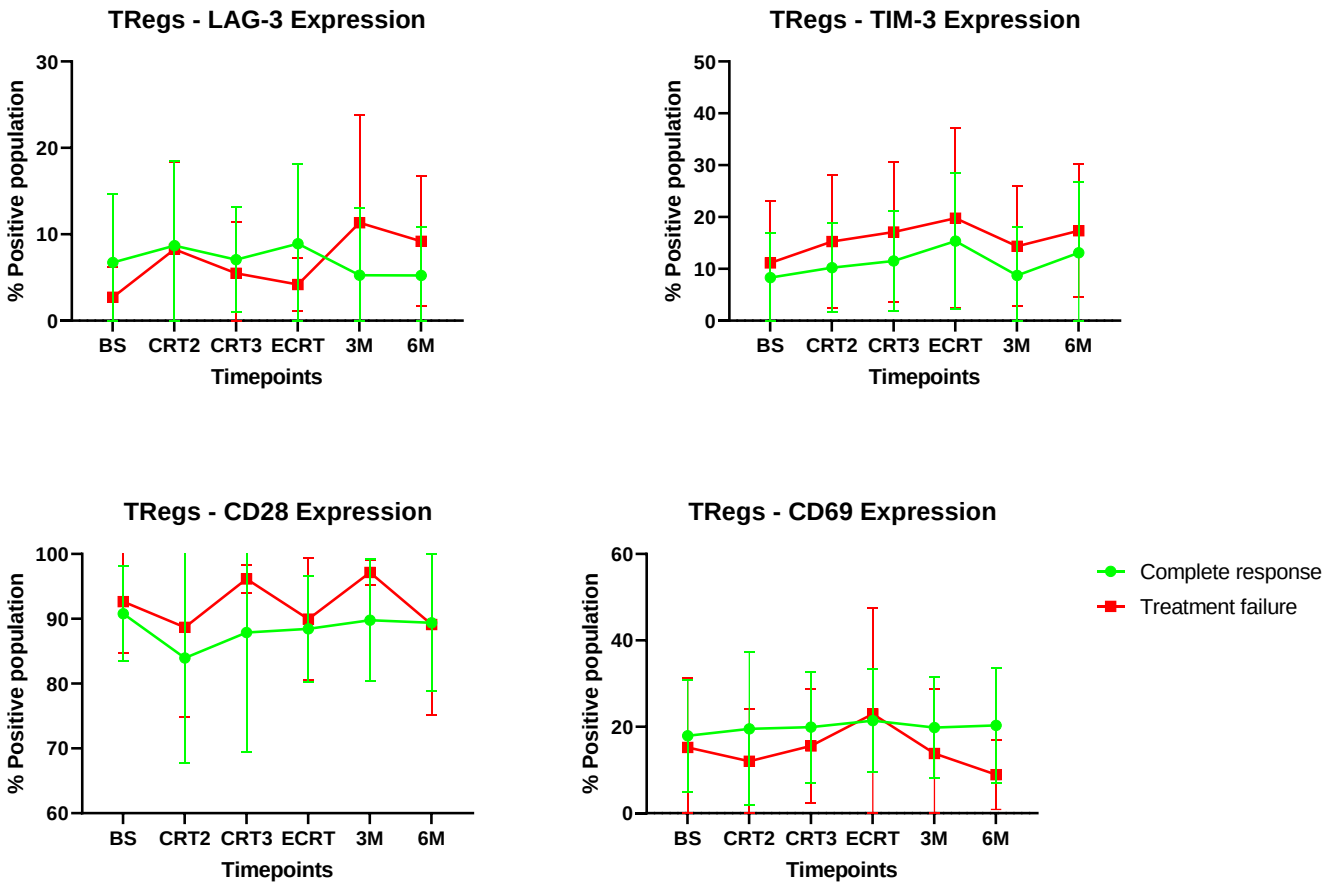


Figure 3.16 Dynamic changes in expression of LAG-3, TIM-3, CD28 and CD69 on Tregs. Data is expressed as % positive of Treg population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

3.9 CD8+ T-cell – targeted markers

Figure 3.17 shows dynamic changes TIGIT, CTLA-4, PD-1 and PD-L1 expression on CD8+ T-cells in treatment failure and complete response groups. Figure 3.18 and Figure 3.19 compare TIGIT, CTLA-4, PD-1 and PD-L1 expression on CD8+ cells at BS, ECRT and 6M for complete response and treatment failure, respectively. There is a statistically significant higher expression of CTLA-4 on CD8+ T-cells at baseline in patients with treatment failure compared to complete response (21.1 % CTLA-4 expression at baseline in treatment failure versus 6.9 % in complete response, adjusted p-value 0.0029), with a trend for this to be maintained at ECRT (14.18 % CTLA-4 expression at ECRT in treatment failure versus 23.67 % in complete response, adjusted p-value 0.332). There is also a trend for higher TIGIT expression at baseline in treatment failure (72.7 % TIGIT expression at baseline in treatment failure versus 46.4 % in complete response, adjusted p-value 0.0663), with a trend for this to be maintained at ECRT (47.37 % TIGIT expression at ECRT in treatment failure versus 67.23 % in complete response, adjusted p-value 0.332).

Figure 3.20 and Figure 3.21 shows changes during and after treatment for these markers within groups. In patients with complete response, there is a statistically significant increase in CTLA-4 from BS to ECRT (6.9 % CTLA-4 expression in complete response at BS to 14.2 % in ECRT, adjusted p-value 0.02), increase in PD-L1 from BS to ECRT (19.9 % PD-L1 expression in complete response at BS to 33 % at ECRT, adjusted p-value 0.0059) and a decrease in PD-L1 from ECRT to 6M (33 % PD-L1 expression in complete response at BS to 19.5 % at ECRT, adjusted p-value 0.02). There was a trend for increase in PD-L1 expression in the treatment failure group from BS to ECRT (26.3 % PD-L1 expression in treatment failure at BS to 40.3 % at ECRT, adjusted p-value 0.165).

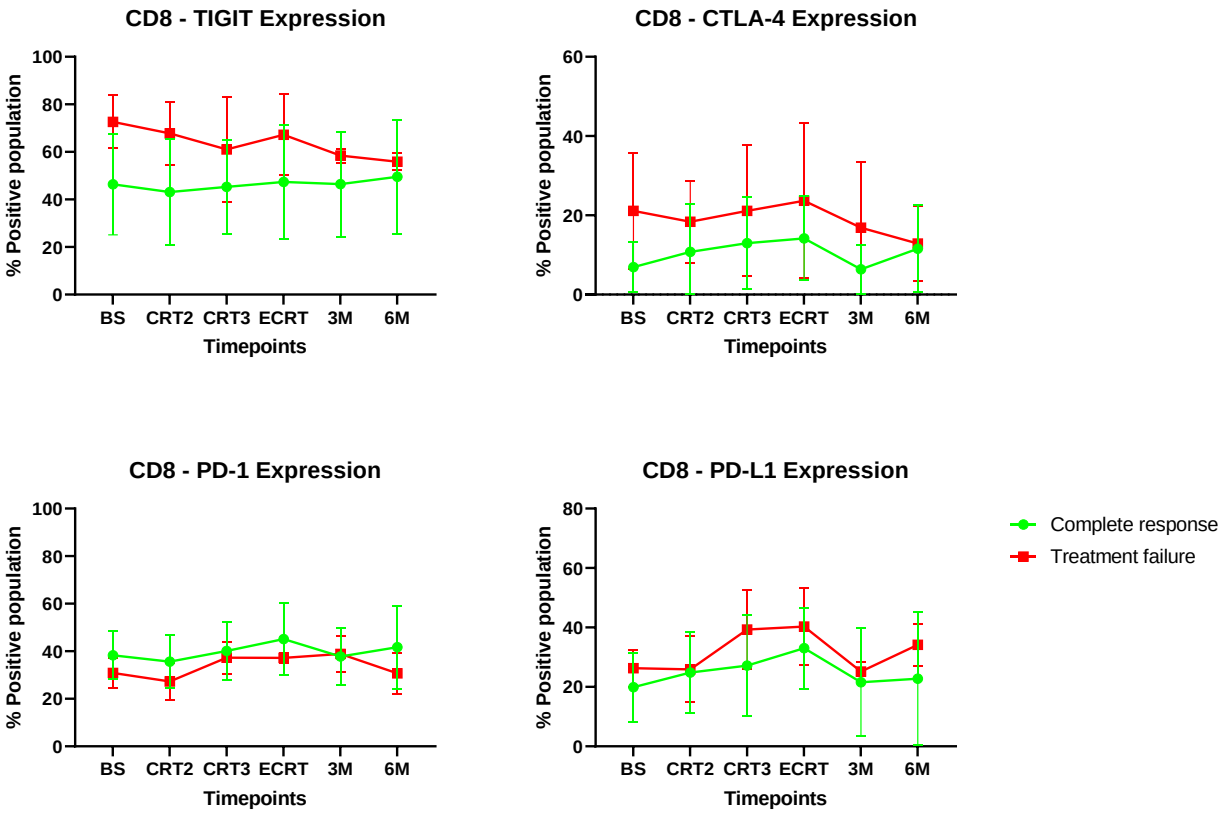


Figure 3.17 Dynamic changes in expression of TIGIT, CTLA-4, PD-1 and PD-L1 Expression on CD8+ T-cells. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

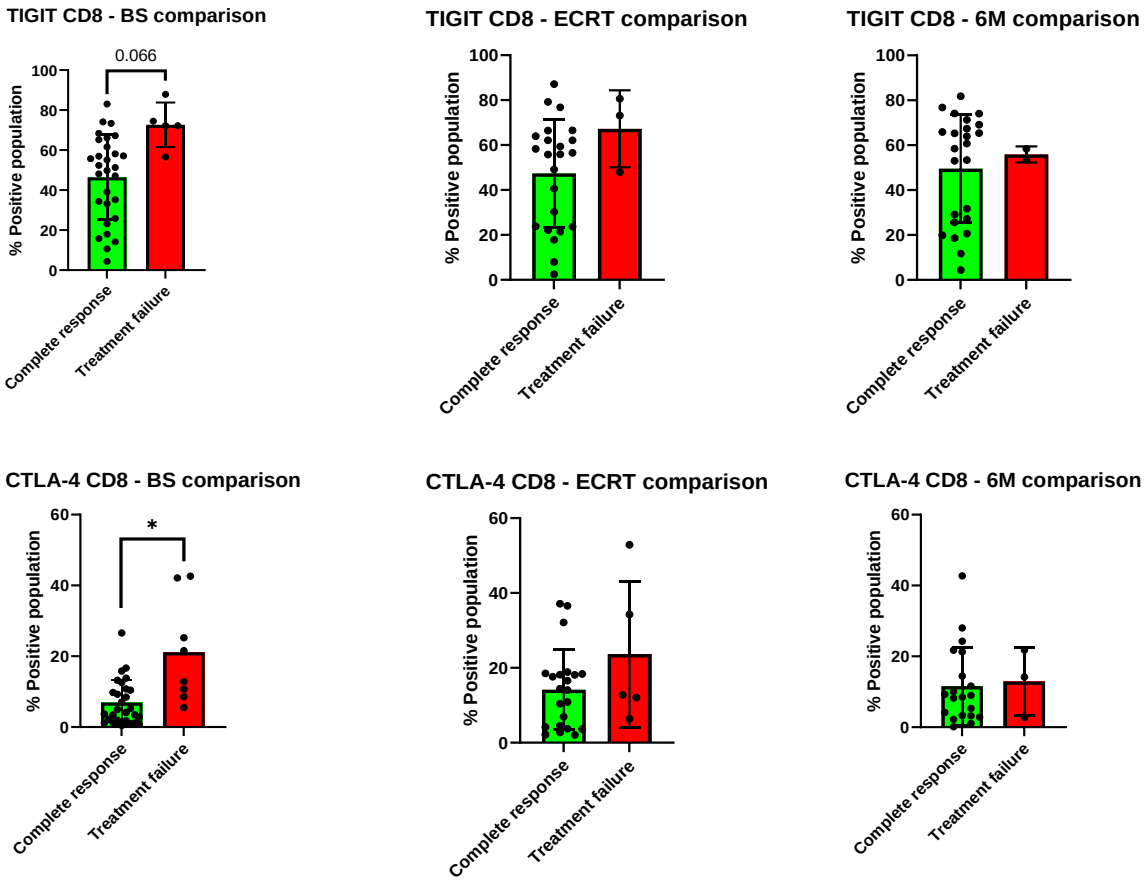


Figure 3.18 Comparison between complete response and treatment failure groups for TIGIT and CTLA-4 on CD8+ T-cells. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

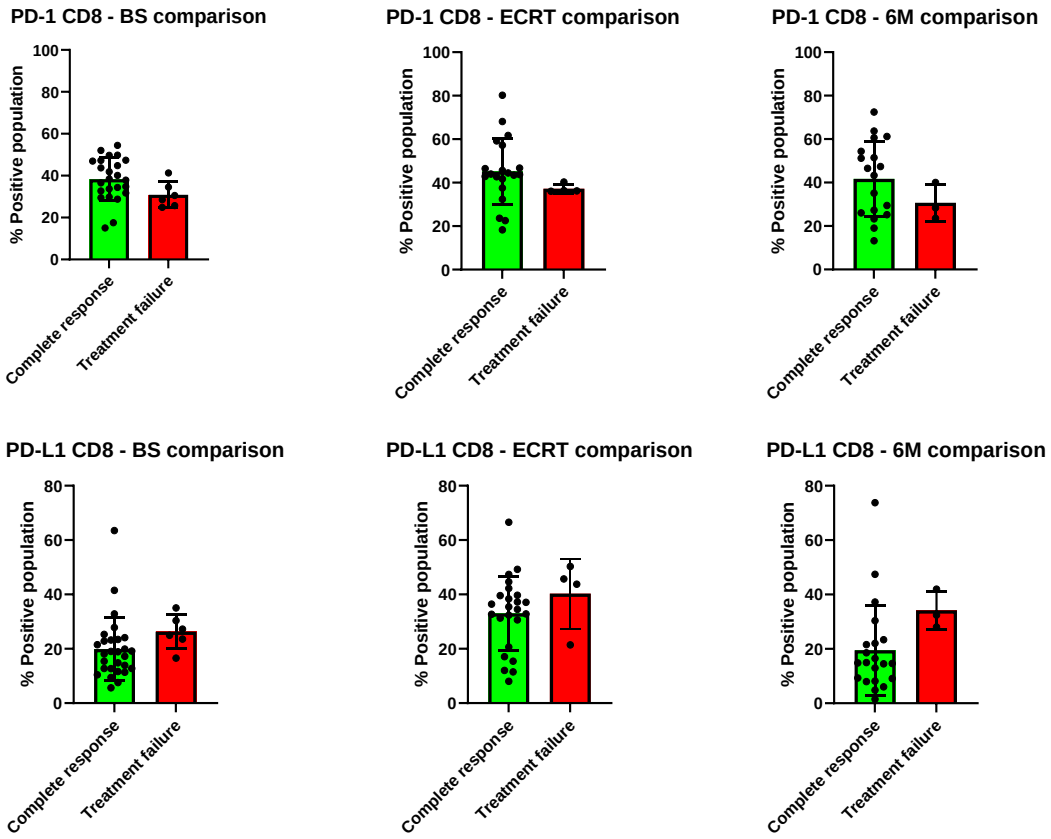


Figure 3.19 Comparison between complete response and treatment failure groups PD-1 and PD-L1 on CD8+ T-cells. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

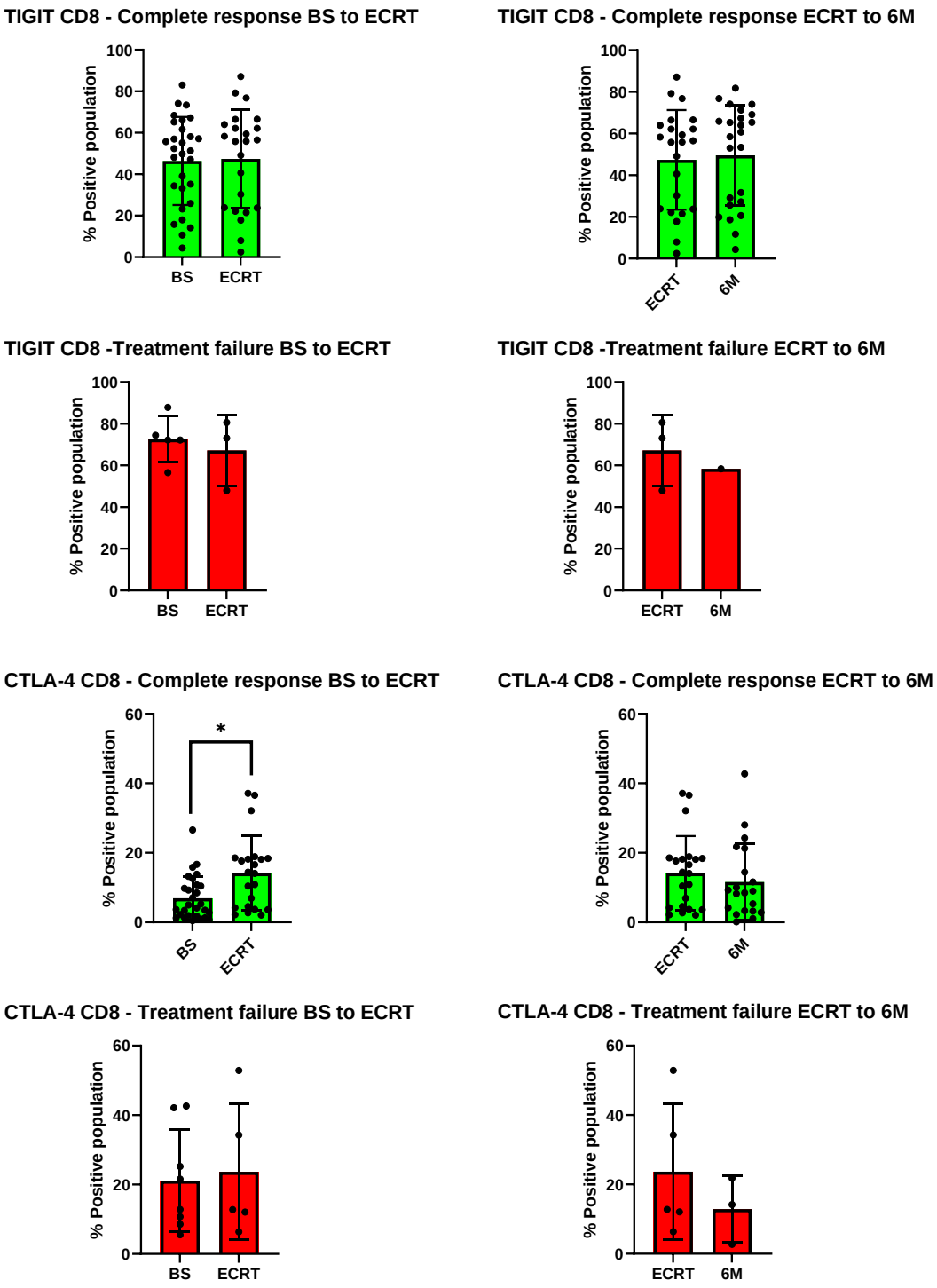


Figure 3.20 Changes in TIGIT and CTLA-4 Expression on CD8+ T-cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05

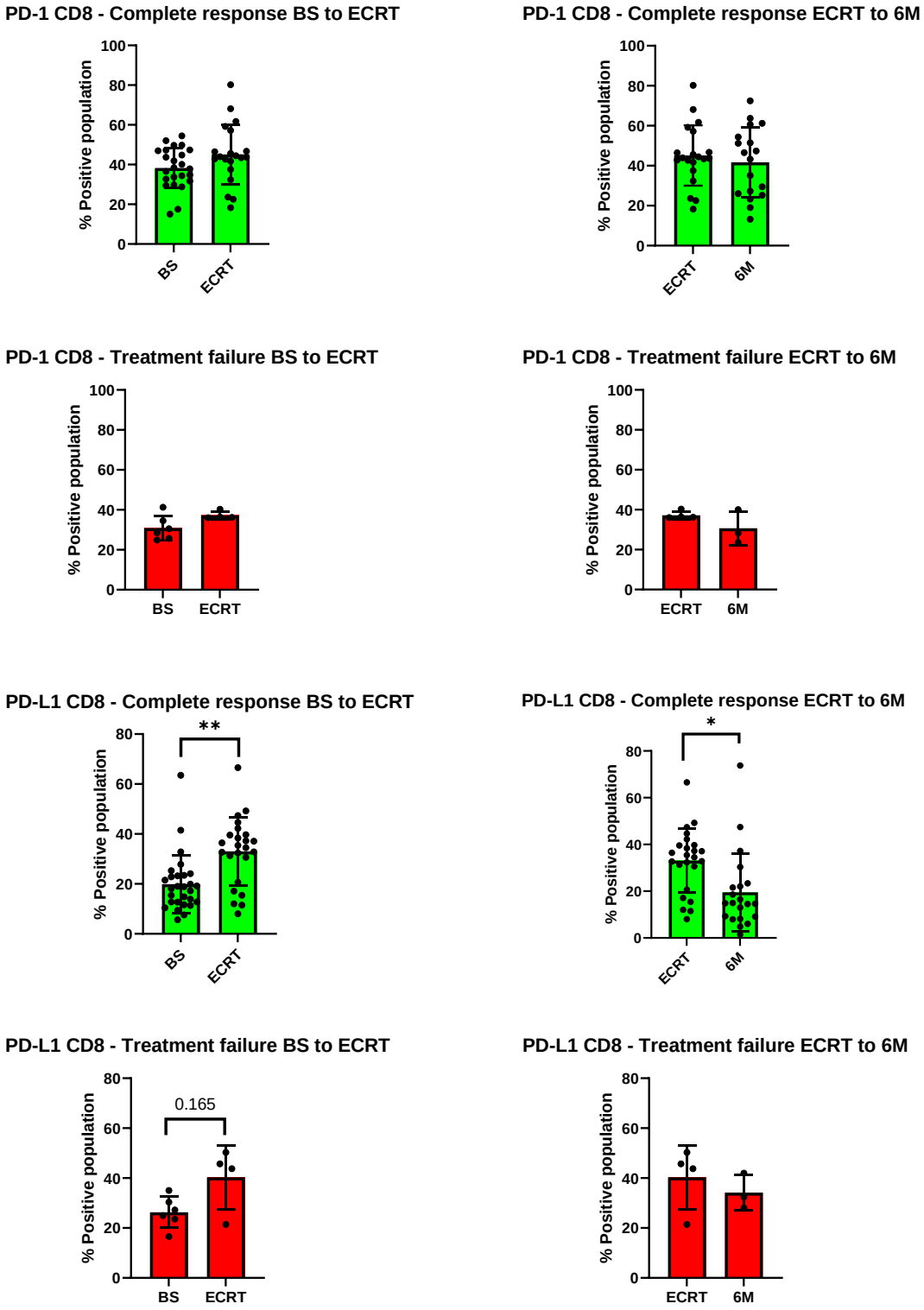


Figure 3.21 Changes in PD-1 and PD-L1 Expression on CD8+ T-cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01

3.10 CD8+ T-cell – exploratory markers

Figure 3.32, Figure 3.23 and Figure 3.24 show dynamic changes in CCR7, OX40, GITR, ICOS, CD40L, BTLA, CD69, CD137, LAG-3, CD25 and TIM-3 expression on CD8 cells in treatment failure and complete response groups, respectively.

There were no statistically significant differences between groups. As shown in

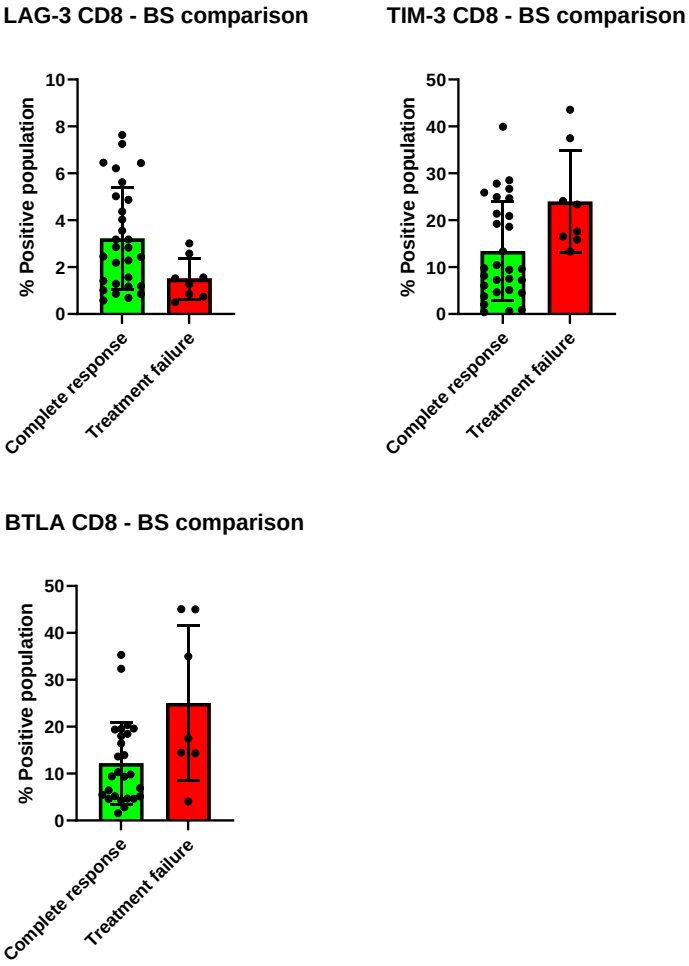


Figure 3.25, there was a trend for higher LAG-3 at baseline in complete responders (3.2 % LAG-3 expression at baseline in treatment failure versus 1.5 % in complete response, adjusted p-value 0.309), higher TIM-3 at baseline in those with treatment failure (24 % TIM-3 expression at baseline in treatment failure versus 13.4 % in complete response, adjusted p-value 0.203) and higher BTLA at baseline in those with treatment failure (25 % BTLA expression in treatment failure versus 12.6 % in complete response, adjusted p-value 0.203).

As shown in Figure 3.26 there was a statistically significant increase in GITR from baseline to ECRT in complete response (8.3 % GITR expression in complete response at

BS to 21.4 % at ECRT, adjusted p-value 0.009). No other changes within groups were statistically significant.

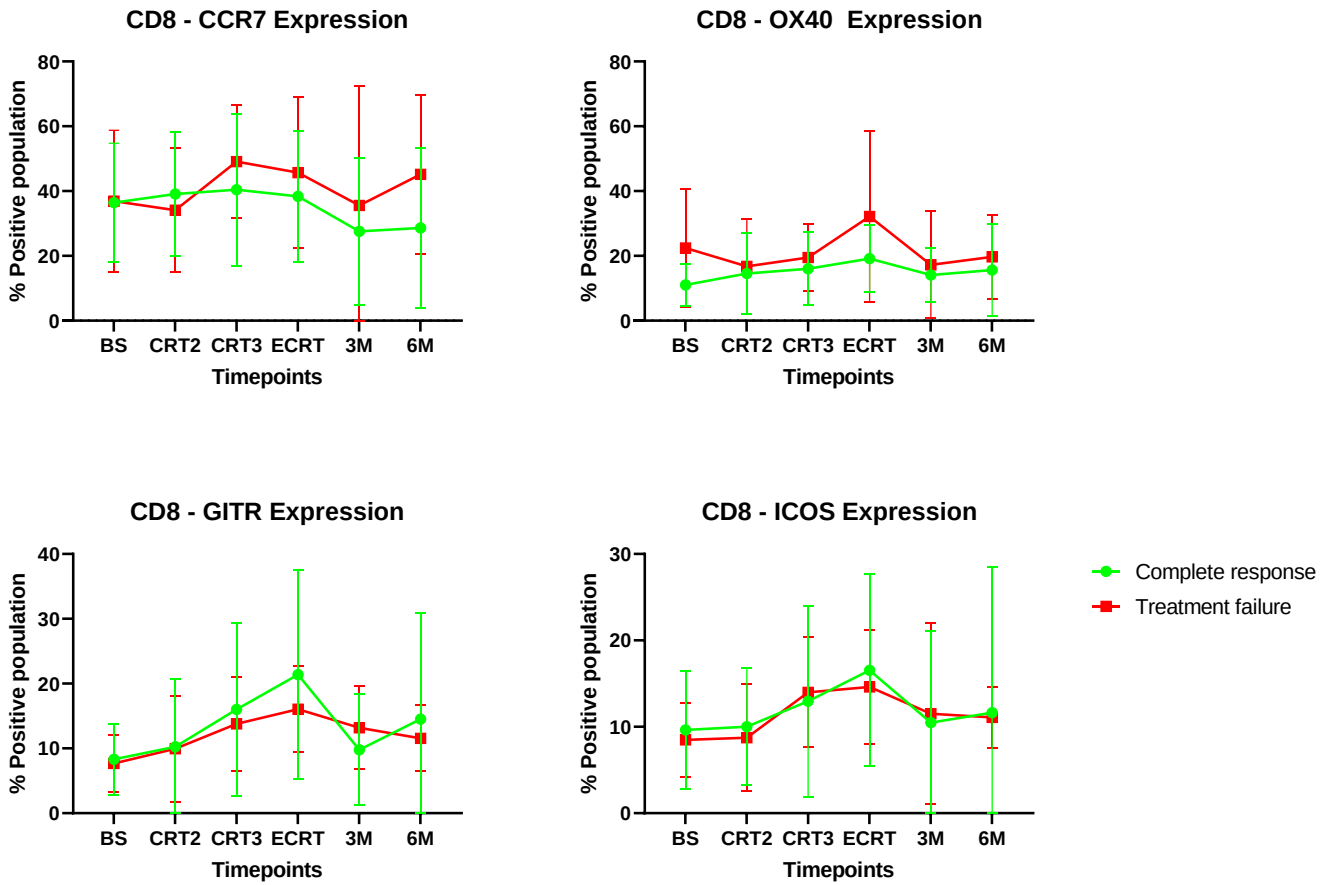


Figure 3.22 Dynamic changes in expression of CCR7, OX40, GITR and ICOS Expression on CD8+ T-cells. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

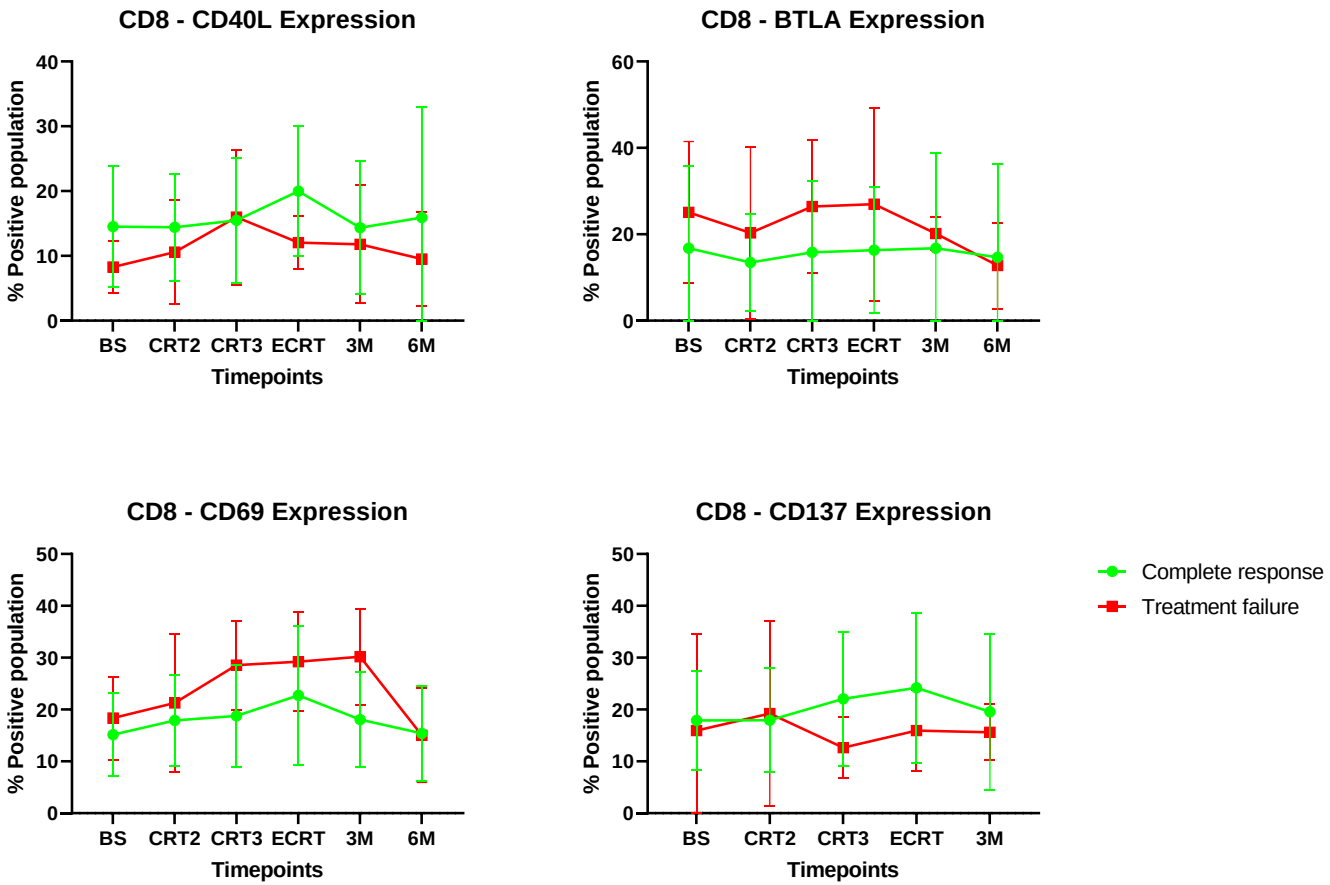


Figure 3.23 Dynamic changes in expression of CD40L, BTLA, CD69 and CD137 Expression on CD8+ T-cells. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

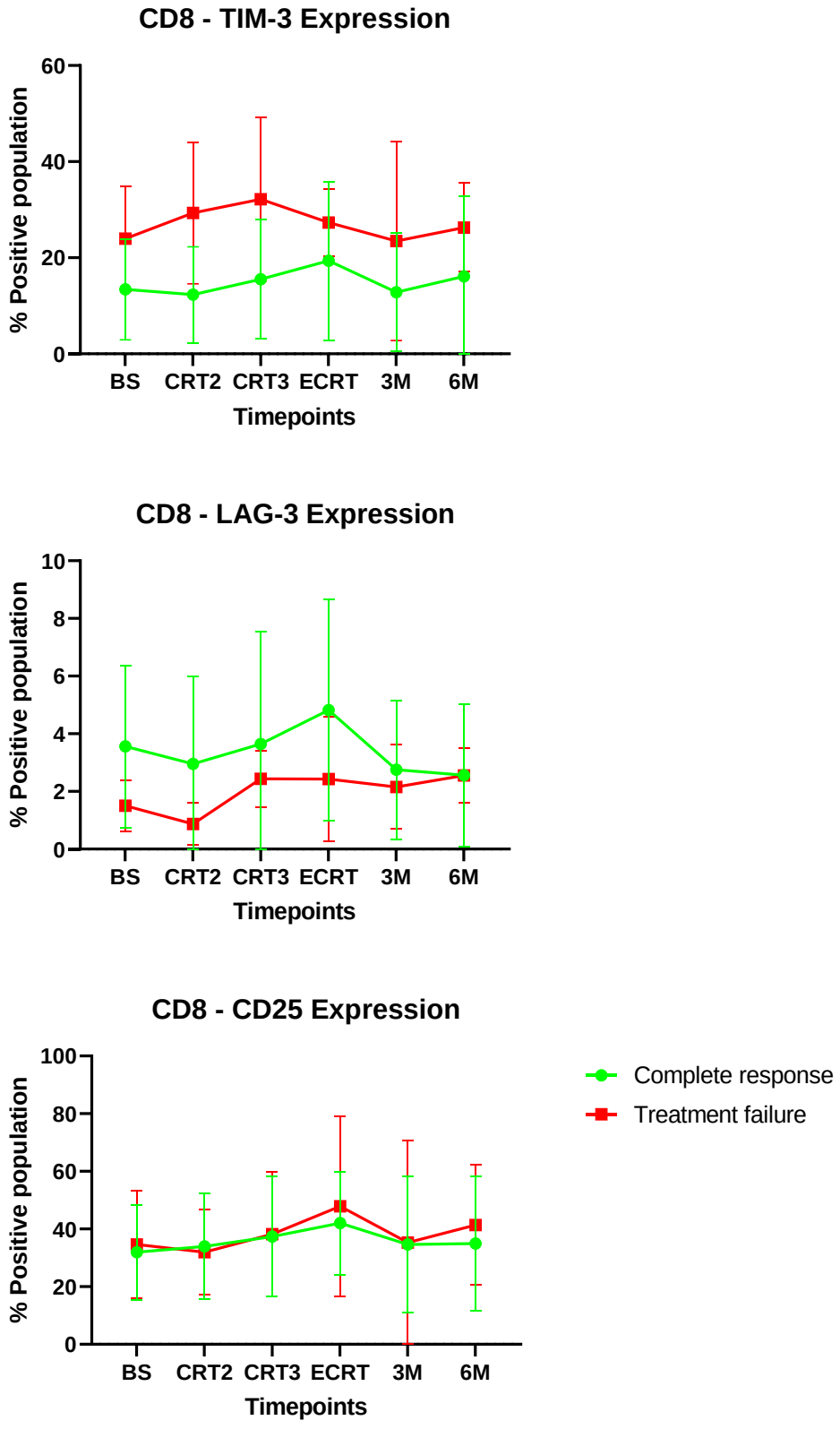


Figure 3.24 Dynamic changes in expression of TIM-3, LAG-3 and CD25 Expression on CD8+ T-cells. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

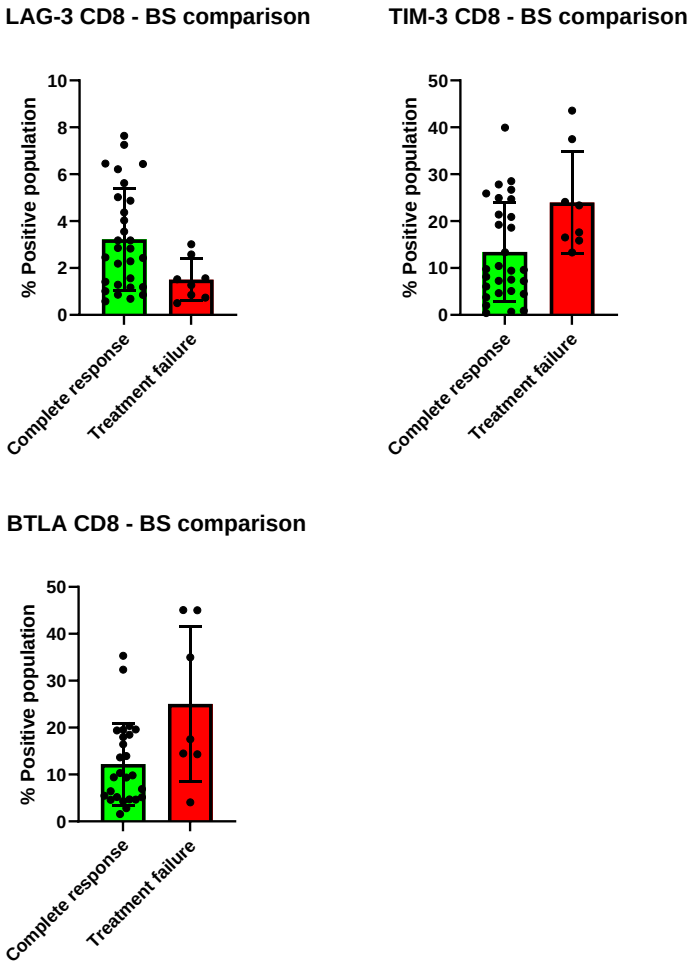


Figure 3.25 LAG-3, TIM-3 and BTLA Expression at Baseline on CD8 T-cells. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=30 (CR); N=8 (TF), sample availability dependent

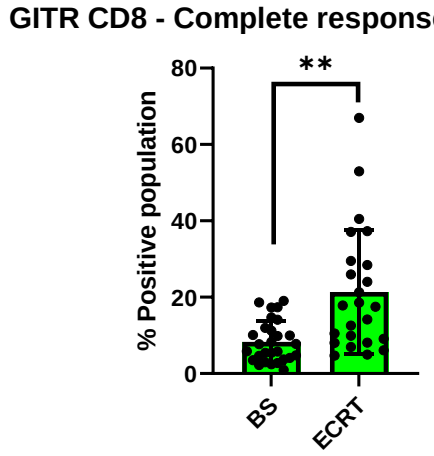


Figure 3.26 GITR Expression on CD8 T-cells from BS to ECRT in complete response. Data is expressed as % positive of CD8+ T-cell population +/-SD. Data is presented for N=30 (BS); N25 (ECRT). ✱✱P ≤ 0.01

3.11 CD4+ T-cell – targeted markers

Figure 3.27 shows dynamic changes TIGIT, CTLA-4, PD-1 and PD-L1 expression on CD4+ T-cells in treatment failure and complete response groups. Figure 3.28 and Figure 3.29 compares TIGIT, CTLA-4, PD-1 and PD-L1 expression on CD8 cells at BS, ECRT and 6M for complete response and treatment failure. There was statistically higher TIGIT expression at baseline for patients with treatment failure (37.2 % TIGIT expression at baseline in treatment failure versus 20.6 % in complete response, adjusted p-value 0.0037), with a trend for this to continue at ECRT (38.48 % TIGIT expression at ECRT in treatment failure versus 27.56 % in complete response, adjusted p-value 0.31). There was statistically higher CTLA-4 expression at baseline for patients with treatment failure (19.8 % CTLA-4 expression at baseline in treatment failure versus 5.3 % in complete response, adjusted p-value 0.0027) with a trend for this to continue at ECRT (18.48 % CTLA-4 expression at ECRT in treatment failure versus 10.72 % in complete response, adjusted p-value 0.31).

Figure 3.30 and Figure 3.31 shows changes during and after treatment for these markers within groups. There was a statistically significant increase in expression from baseline to ECRT for patients with complete response for TIGIT (20.56 % TIGIT expression in complete response at BS to 27.56 % at ECRT, adjusted p-vale 0.046), CTLA-4 (5.32 % CTLA-4 expression in complete response at BS to 10.72 % at ECRT, adjusted p-value 0.009), PD-1 (26.97 % PD-1 expression in complete response at BS to 42.97 % at ECRT, adjusted p-value <0.00001) and PD-L1 (23.03 % PD-L1 expression in complete response at BS to 41.49 % at ECRT, adjusted p-value <0.00001), with a statistically significant increase in PD-L1 from baseline to ECRT also present for treatment failure (27.64 % PD-L1 expression in treatment failure at BS to 34.51 % at ECRT, adjusted p-value 0.023).

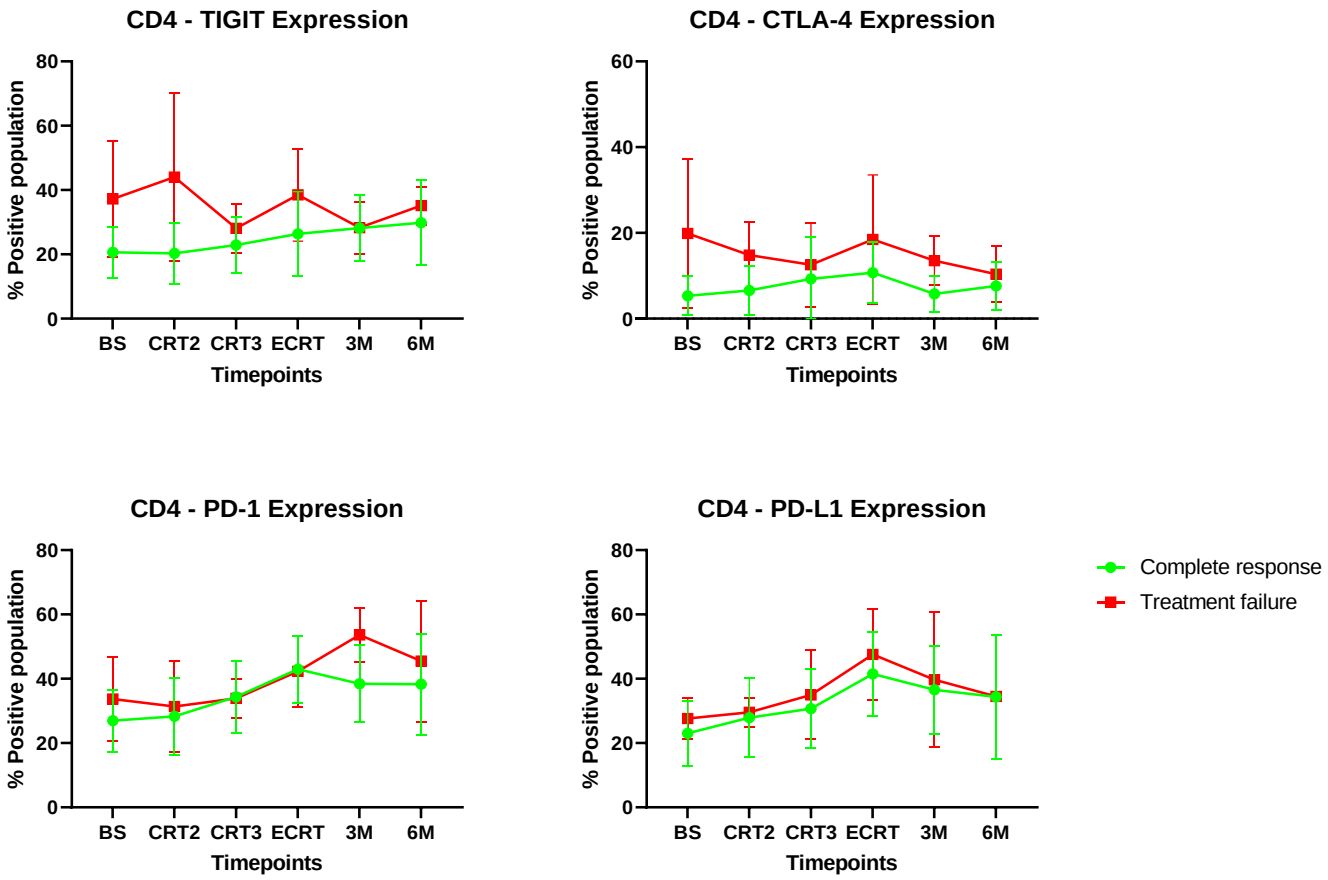


Figure 3.27 Dynamic changes in expression of TIGIT, CTLA-4, PD-1 and PD-L1 Expression on CD4+ T-cells. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

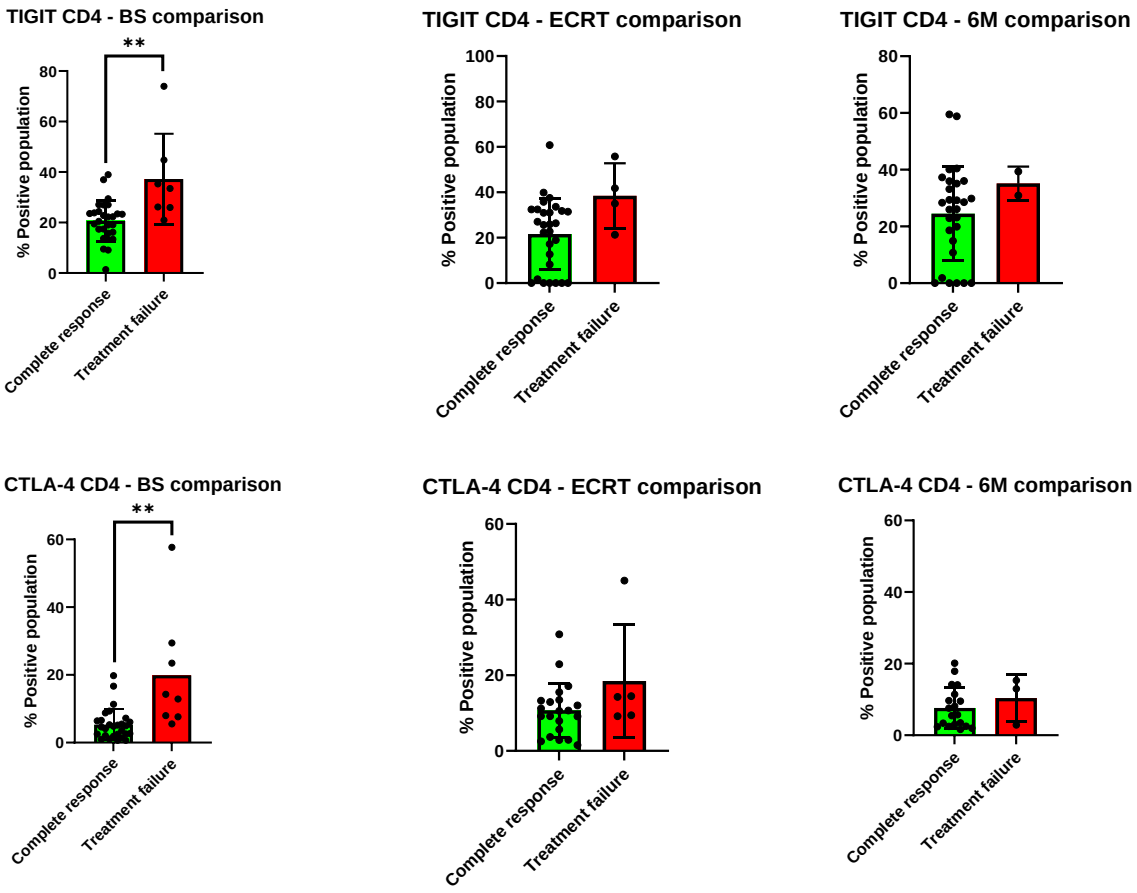


Figure 3.28 Comparison between complete response and treatment failure groups for TIGIT and CTLA-4 on CD4+ T-cells. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱✱P ≤ 0.01

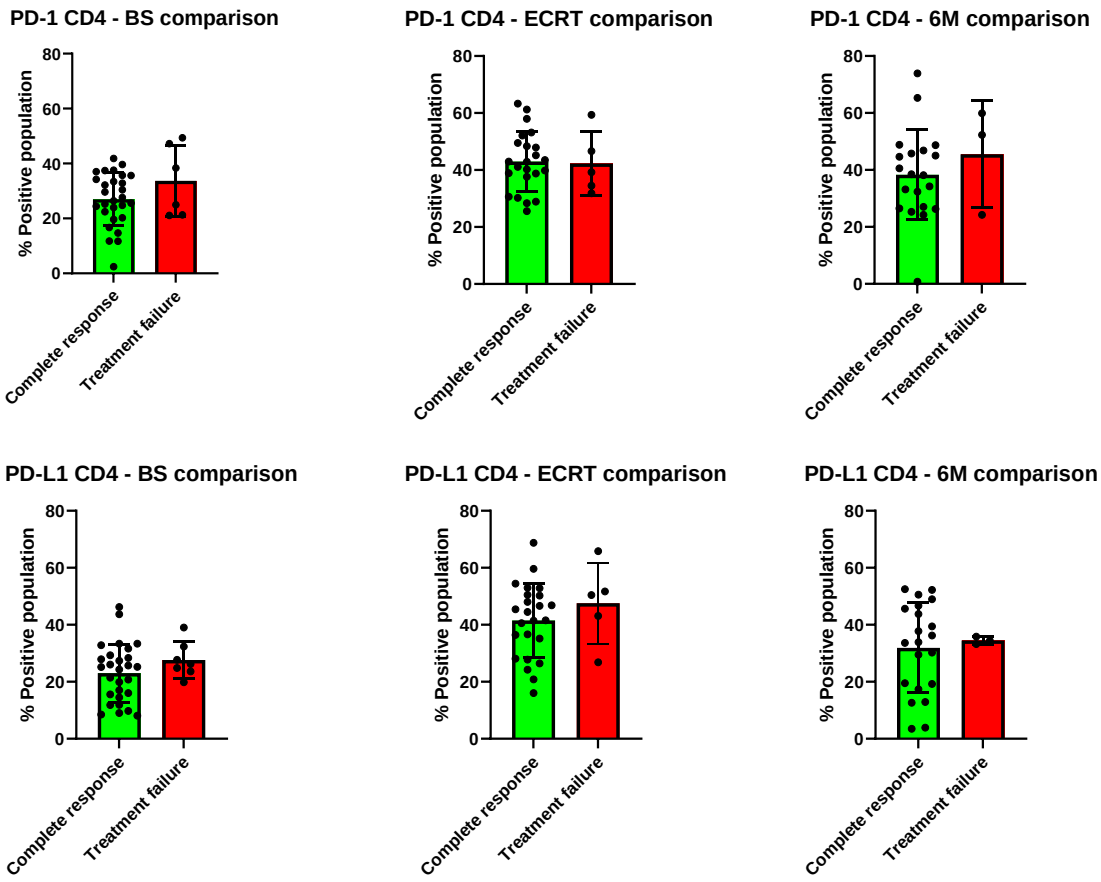


Figure 3.29 Comparison between complete response and treatment failure groups for PD-1 and PD-L1 on CD4+ T-cells. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

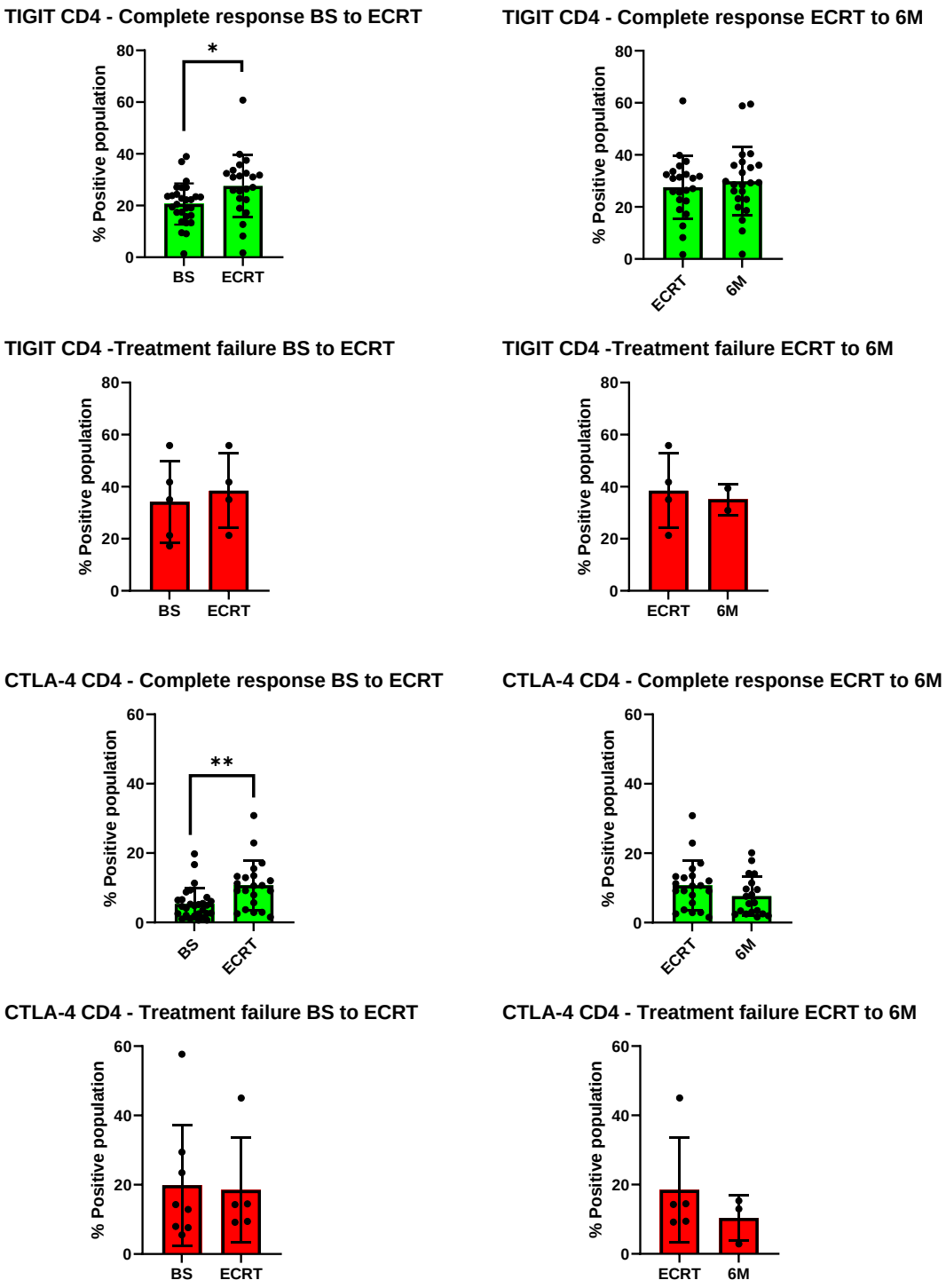


Figure 3.30 Changes in TIGIT and CTLA-4 Expression on CD4+ T-cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01

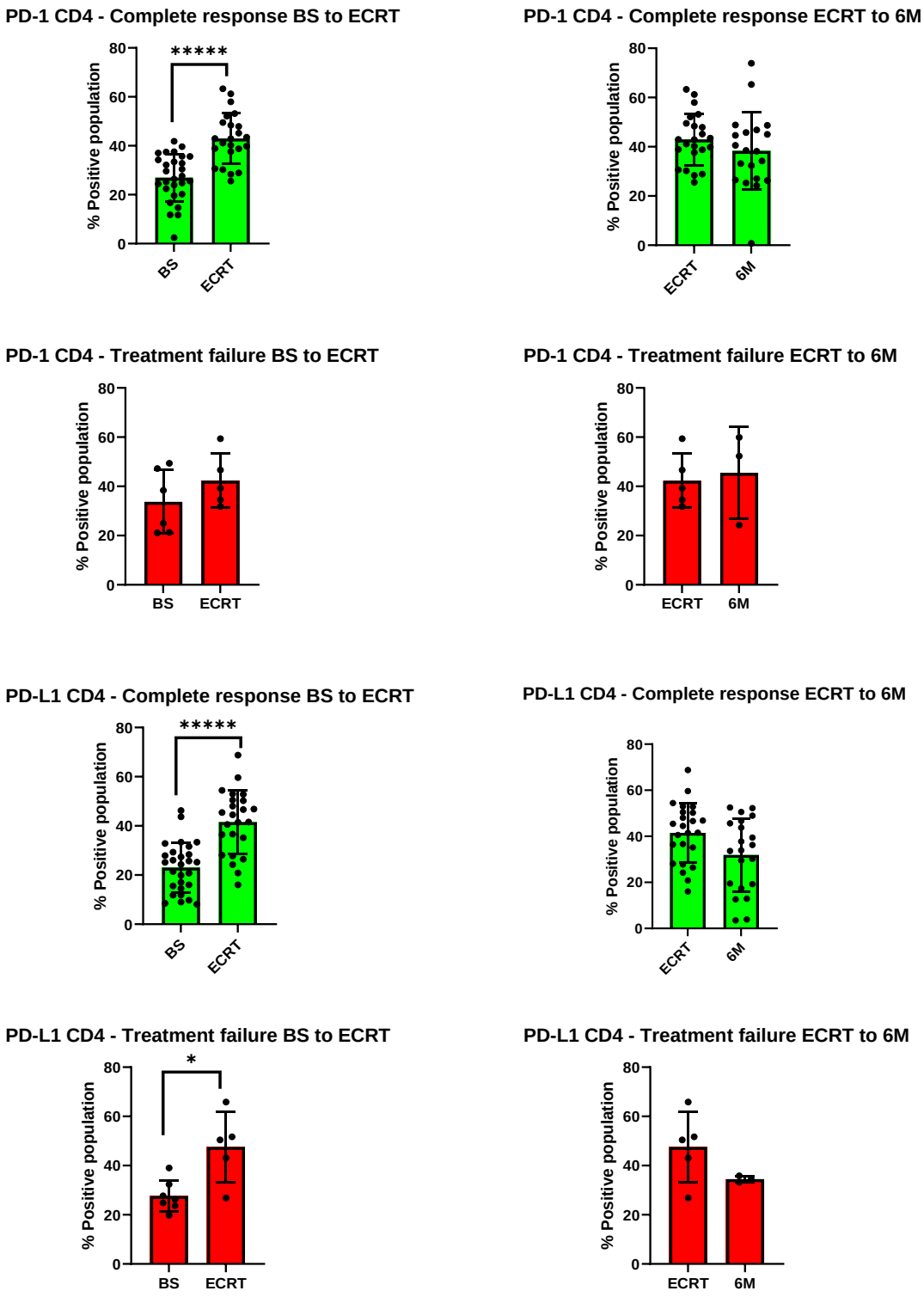


Figure 3.31 Changes in PD-1 and PD-L1 Expression on CD4+ T-cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱✱✱✱P ≤ 0.00001

3.12 CD4+ T-cell – exploratory markers

Figure 3.32, Figure 3.33 and Figure 3.34 show dynamic changes CCR7, OX40, GITR, ICOS, CD40L, BTLA, CD69, CD137, LAG-3, CD25 and TIM-3 expression on CD4 cells in treatment failure and complete response groups respectively.

There were no significant differences between groups. As shown in Figure 3.35, there was an increase from baseline to ECRT in complete response for OX40 (22.4 % OX40 expression in complete response at BS to 39.9 % at ECRT, adjusted p-value 0.0001), GITR (7.3 % GITR expression in complete response at BS to 21.3 % at ECRT, adjusted p-value 0.0006) and CD25 (54.9 % CD25 expression in complete response at BS to 66 % at ECRT, adjusted p-value 0.042). Similar trends were seen in ICOS from BS to ECRT in complete response (10.78 % ICOS expression in complete response at BS to 18.37 % at ECRT, adjusted p-value 0.058) and treatment failure (8.77 % ICOS expression in treatment failure at BS to 16.72 % at ECRT, adjusted p-value 0.409), CD40L from BS to ECRT in complete response (9.96 % CD40L expression in complete response at BS to 14.93 % at ECRT, adjusted p-value 0.147), and TIM-3 from BS to ECRT in complete response (7.62 % TIM-3 expression in complete response at BS to 12.96 % at ECRT, adjusted p-value 0.063).

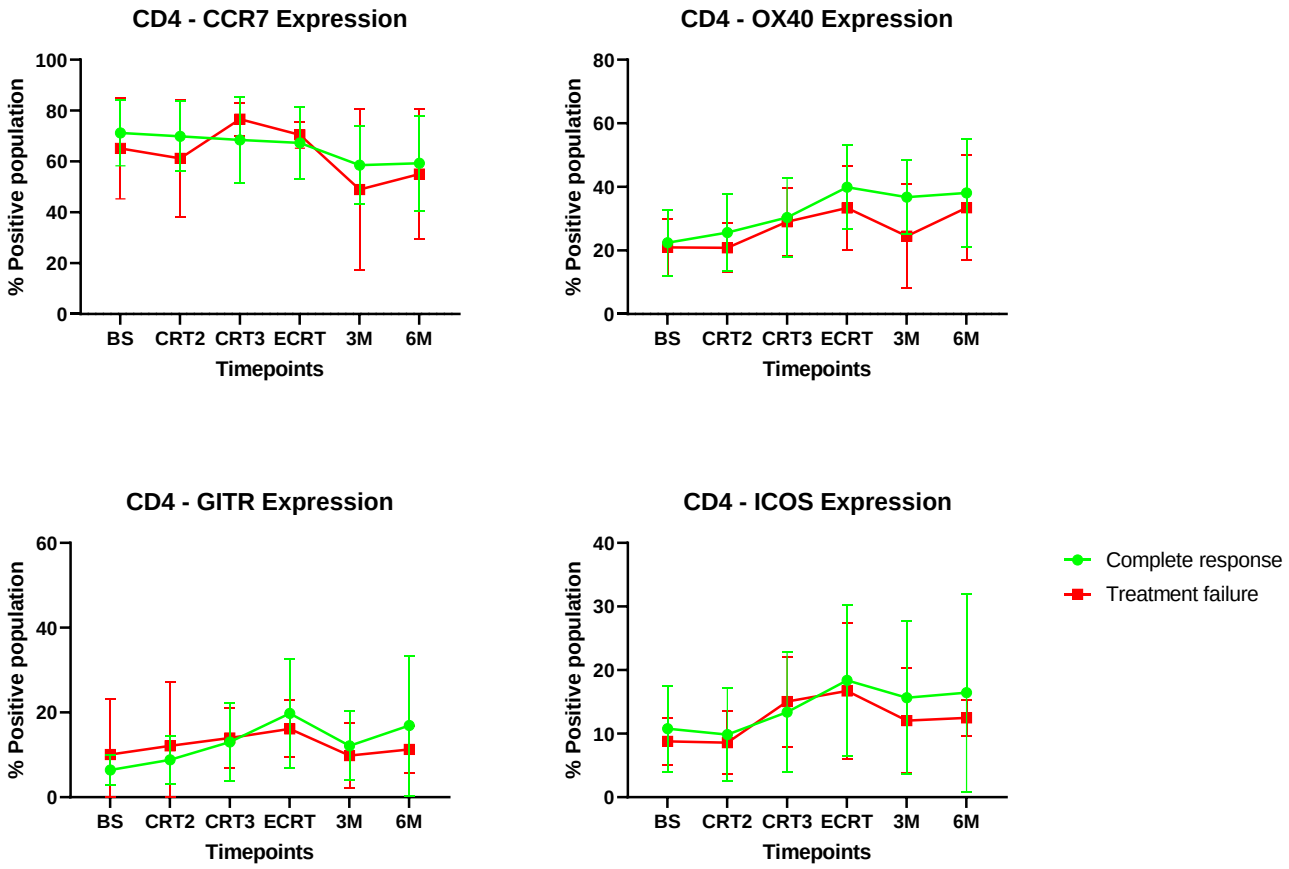


Figure 3.32 Dynamic changes in expression of CCR7, OX40, GITR and ICOS Expression on CD4+ T-cells. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

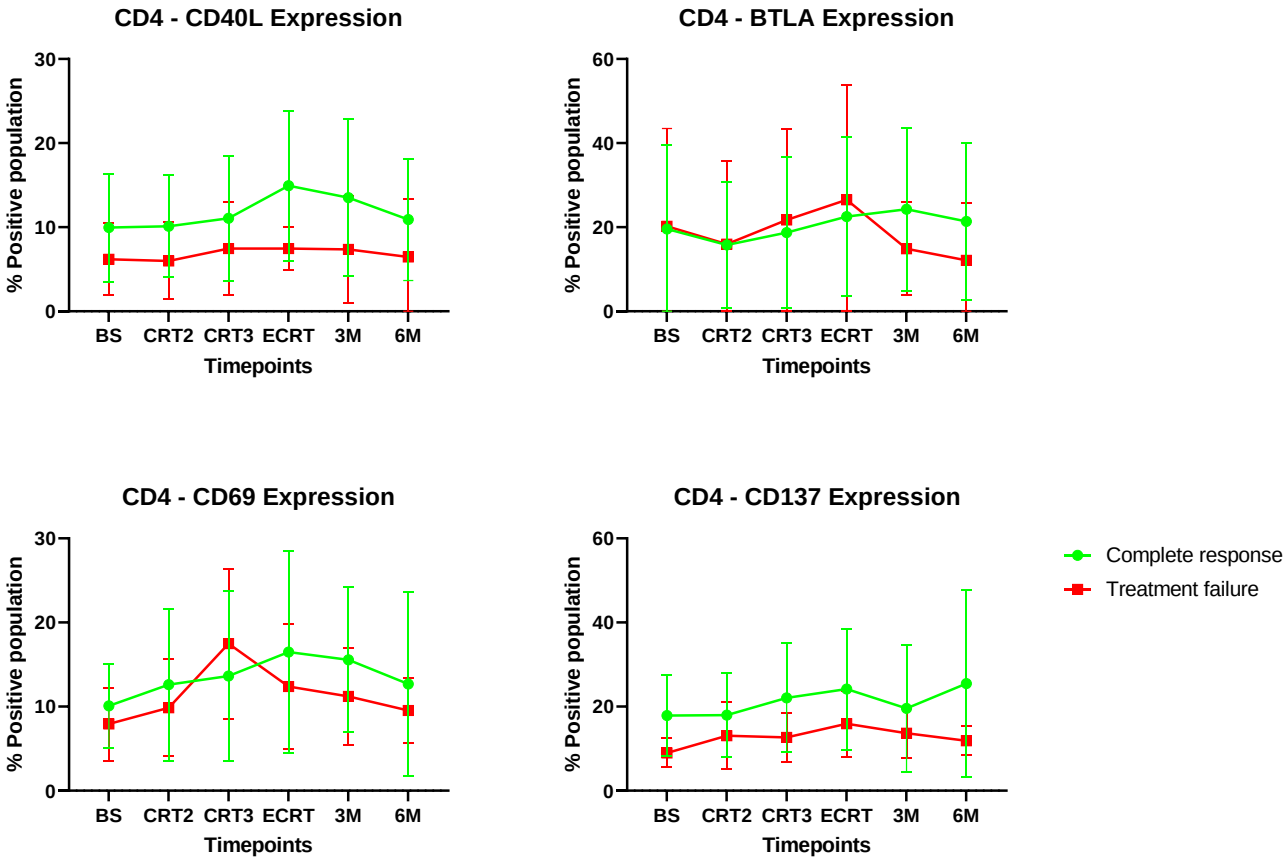


Figure 3.33 Dynamic changes in expression of CD40L, BTLA, CD69 and CD137 Expression on CD4+ T-cells. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

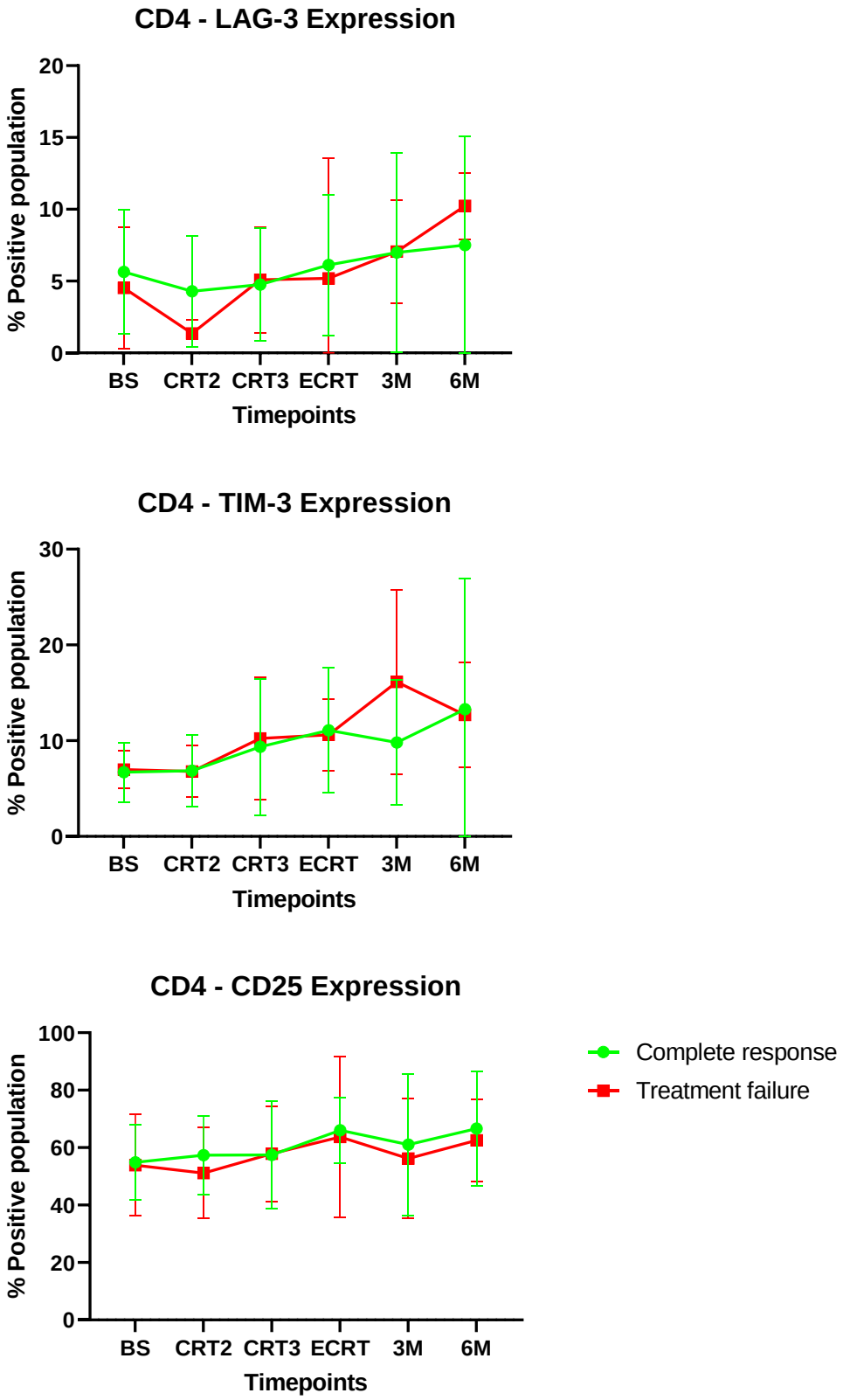


Figure 3.34 Dynamic changes in expression of LAG-3, TIM-3 and CD25 Expression on CD4+ T-cells. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

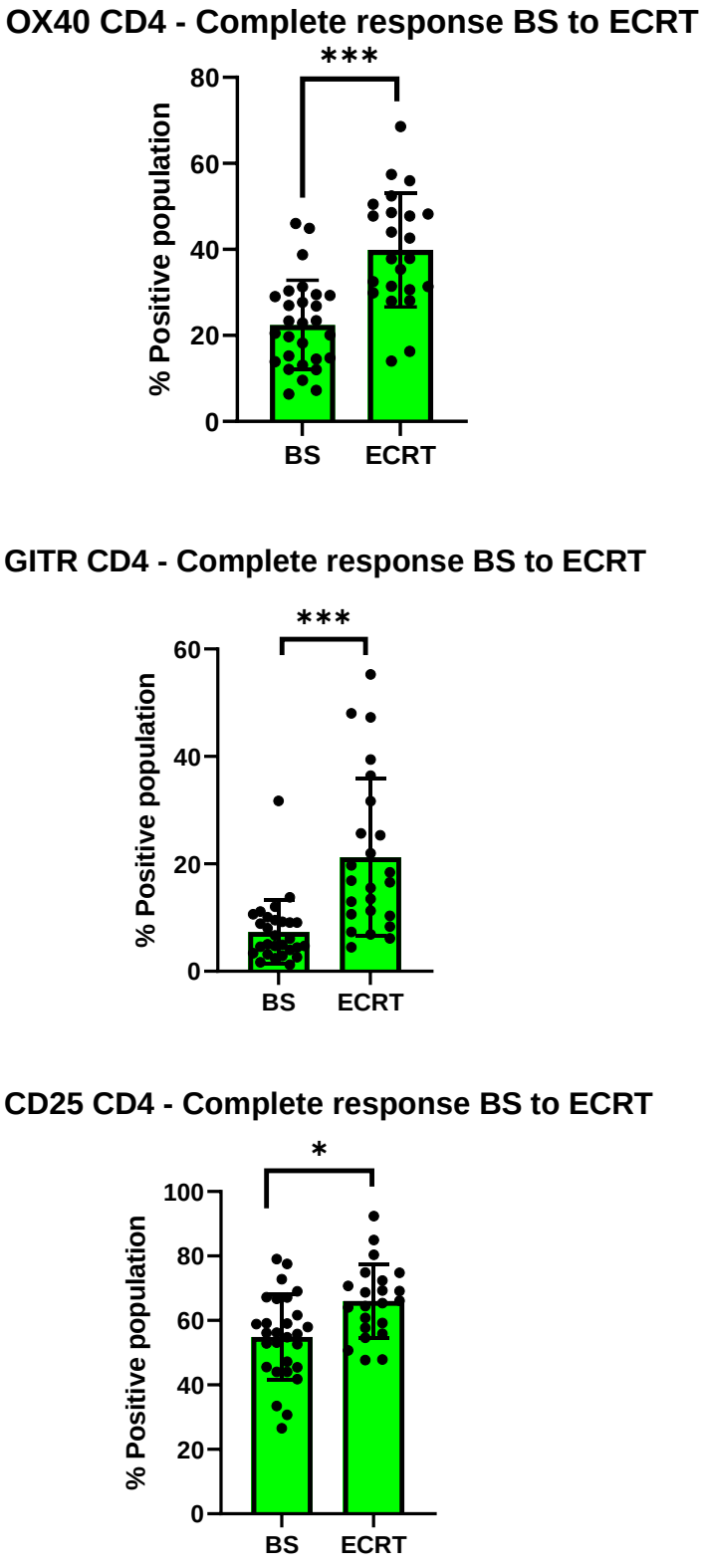


Figure 3.35 Changes in in OX40, GITR and CD25 Expression on CD4+ T-cells from baseline to end of treatment for complete response. Data is expressed as % positive of CD4+ T-cell population +/-SD. Data is presented for N=30 (BS); N=25 (ECRT), sample availability dependent. ✱P ≤ 0.05, ✱✱✱P ≤ 0.001

3.13 NK cell – targeted markers

Figure 3.36 show dynamic changes in TIGIT, CTLA-4, PD-1 and PD-L1 expression on NK cells in treatment failure and complete response groups, respectively. Figure 3.37 and Figure 3.38 compare TIGIT, CTLA-4, PD-1 and PD-L1 expression on NK cells at BS, ECRT and 6M for complete response and treatment failure. After adjustment, there were no statistically significant differences between groups. However, there was a trend for higher TIGIT expression at baseline for patients with treatment failure compared to complete response (59.2 % TIGIT expression at baseline in treatment failure versus 38.7 %, adjusted p-value 0.549). There was a trend for this difference in TIGIT between the groups to be maintained at ECRT (54.25 % TIGIT expression at ECRT in treatment failure versus 41.19 % in complete response, adjusted p-value 0.549) and 6M (64.37 % TIGIT expression at 6M in treatment failure versus 43.69 % in complete response, adjusted p-value 0.549).

Figure 3.39 and Figure 3.40 show changes during and after treatment for these markers within groups. Within groups, there was a statistically significant increase from BS to ECRT for CTLA-4 for complete response (4.44 % CTLA-4 expression in complete response at BS to 13.13 % at ECRT, adjusted p-value 0.007) and treatment failure (3.25 % CTLA-4 expression in treatment failure at BS to 12.55 % at ECRT, adjusted p-value 0.049), PD-1 for complete response (16.83 % PD-1 expression in complete response at BS to 38.46 % at ECRT, adjusted p-value 0.00003) and treatment failure (10.57 % PD-1 expression in treatment failure at BS to 38.63 % at ECRT, adjusted p-value 0.015) and PD-L1 for complete response (5.89 % PD-L1 expression in complete response at BS to 19.02 % at ECRT, adjusted p-value 0.0002) and treatment failure (6.1 % PD-L1 expression in treatment failure at BS to 25.2 % at ECRT, adjusted p-value 0.0069). There was a statistically significant decrease from ECRT to 6M in the complete response group for CTLA-4 (13.13 % CTLA-4 expression in complete response at ECRT to 5.21% at 6M, adjusted p-value 0.025), PD-1 (38.46 % PD-1 expression in complete response at ECRT to 21.96 % at 6M, adjusted p-value 0.0063) and PD-L1 (17.45 % PD-L1 expression in complete response at ECRT to 6.88 % at 6M, adjusted p-value 0.0024), with similar trends seen in the treatment failure group. In contrast there was minimal change in TIGIT expression in response to treatment.

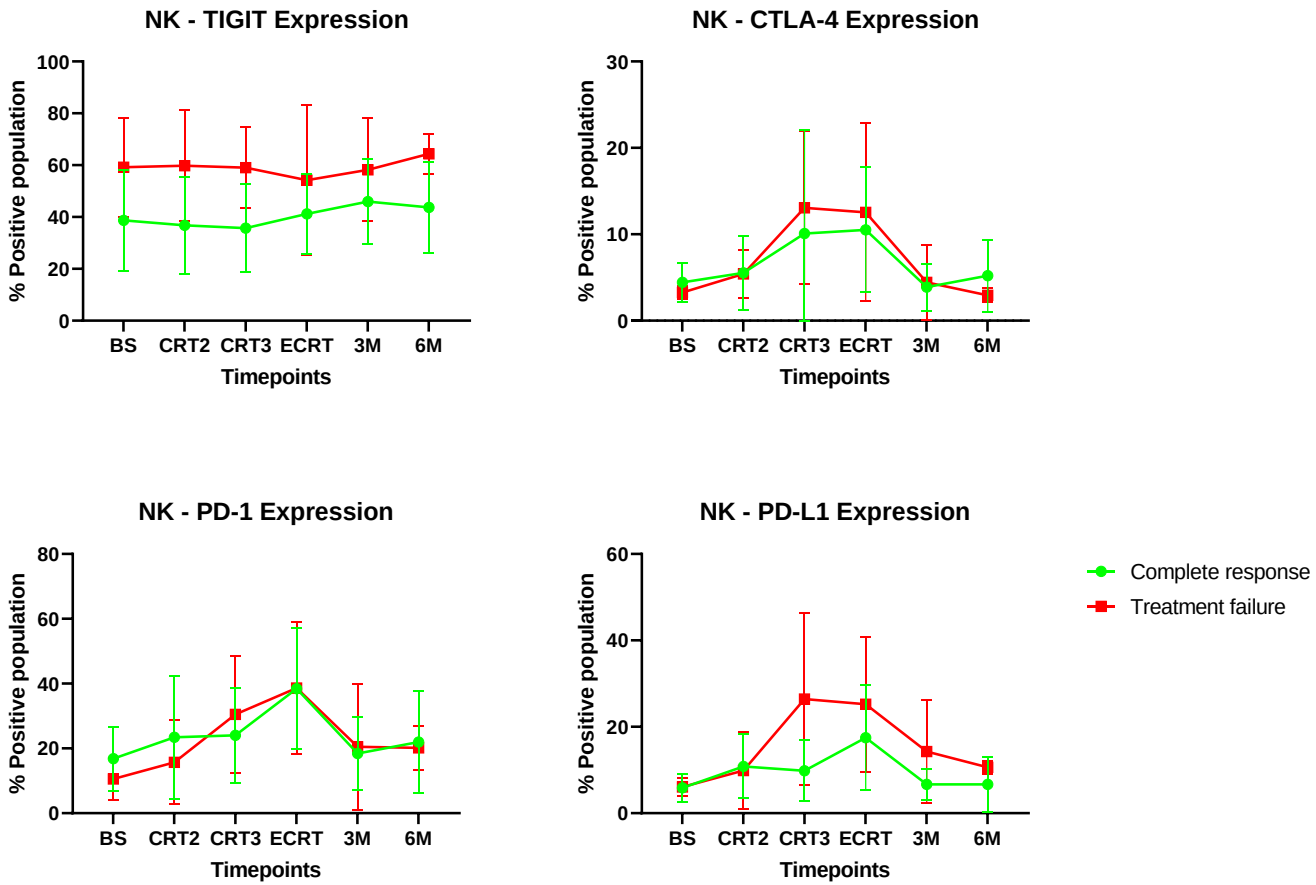


Figure 3.36 Dynamic changes in expression of TIGIT, CTLA-4, PD-1, and PD-L1 Expression on NK cells. Data is expressed as % positive of NK cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

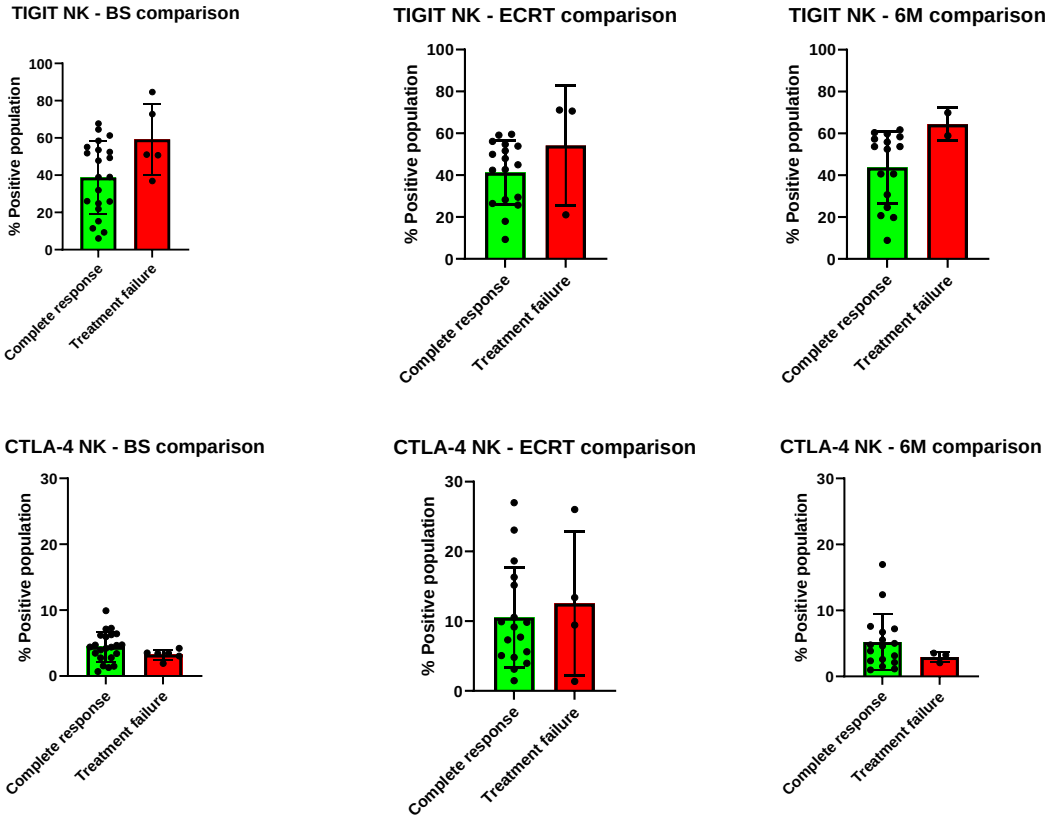


Figure 3.37 Comparison between complete response and treatment failure groups for TIGIT and CTLA-4 on Natural Killer cells. Data is expressed as % positive of Natural Killer cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

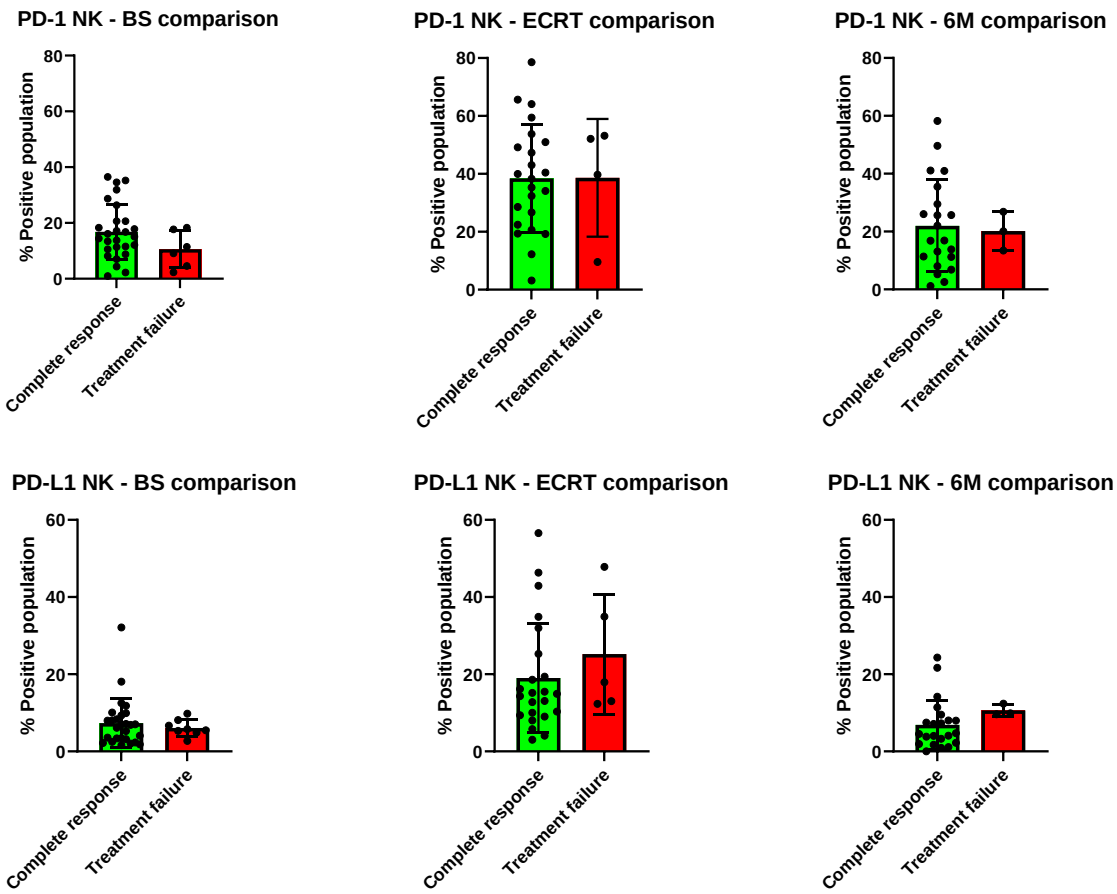


Figure 3.38 Comparison between complete response and treatment failure groups for PD-1 and PD-L1 on Natural Killer cells. Data is expressed as % positive of Natural Killer cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

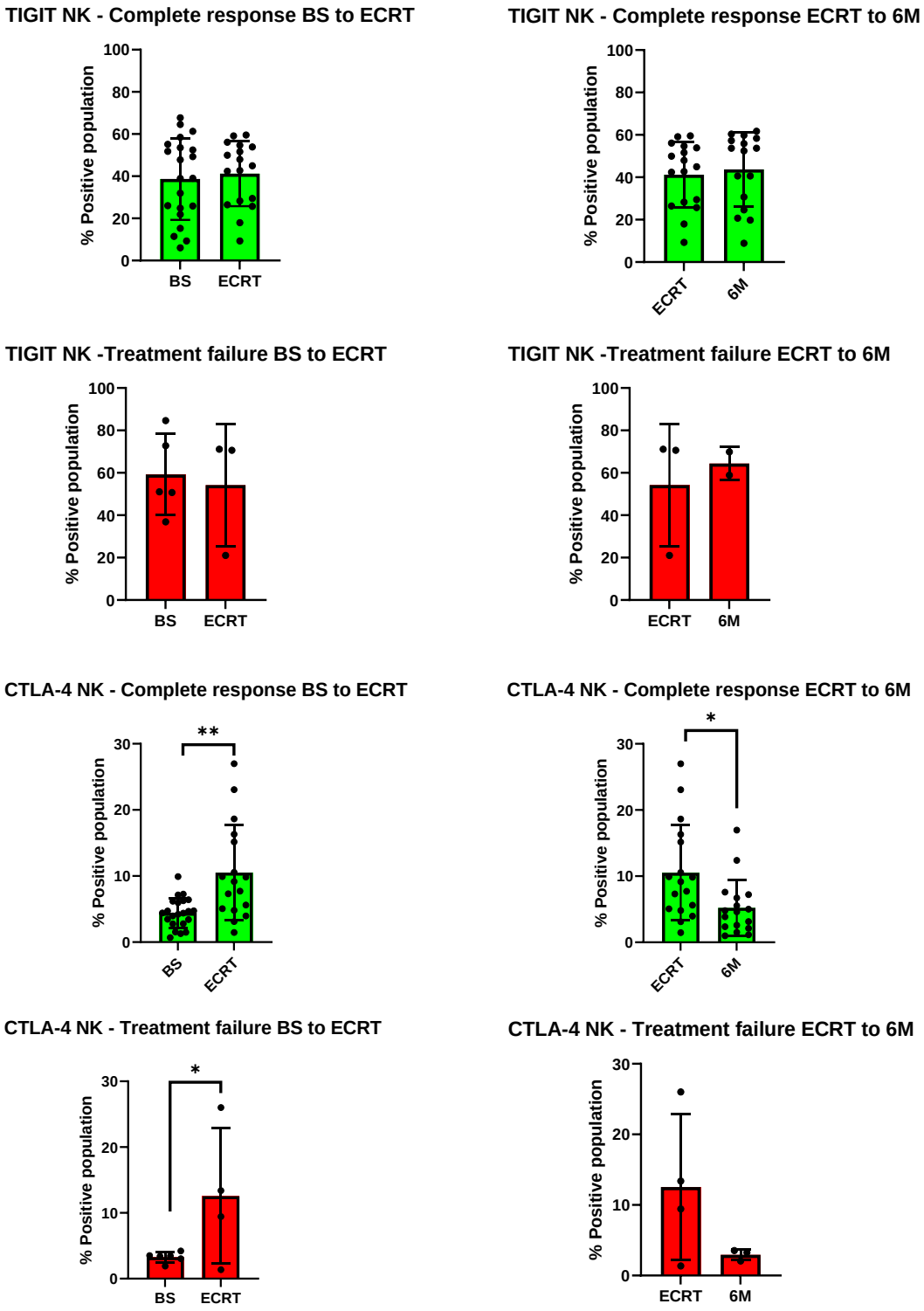


Figure 3.39 Changes in TIGIT and CTLA-4 Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01

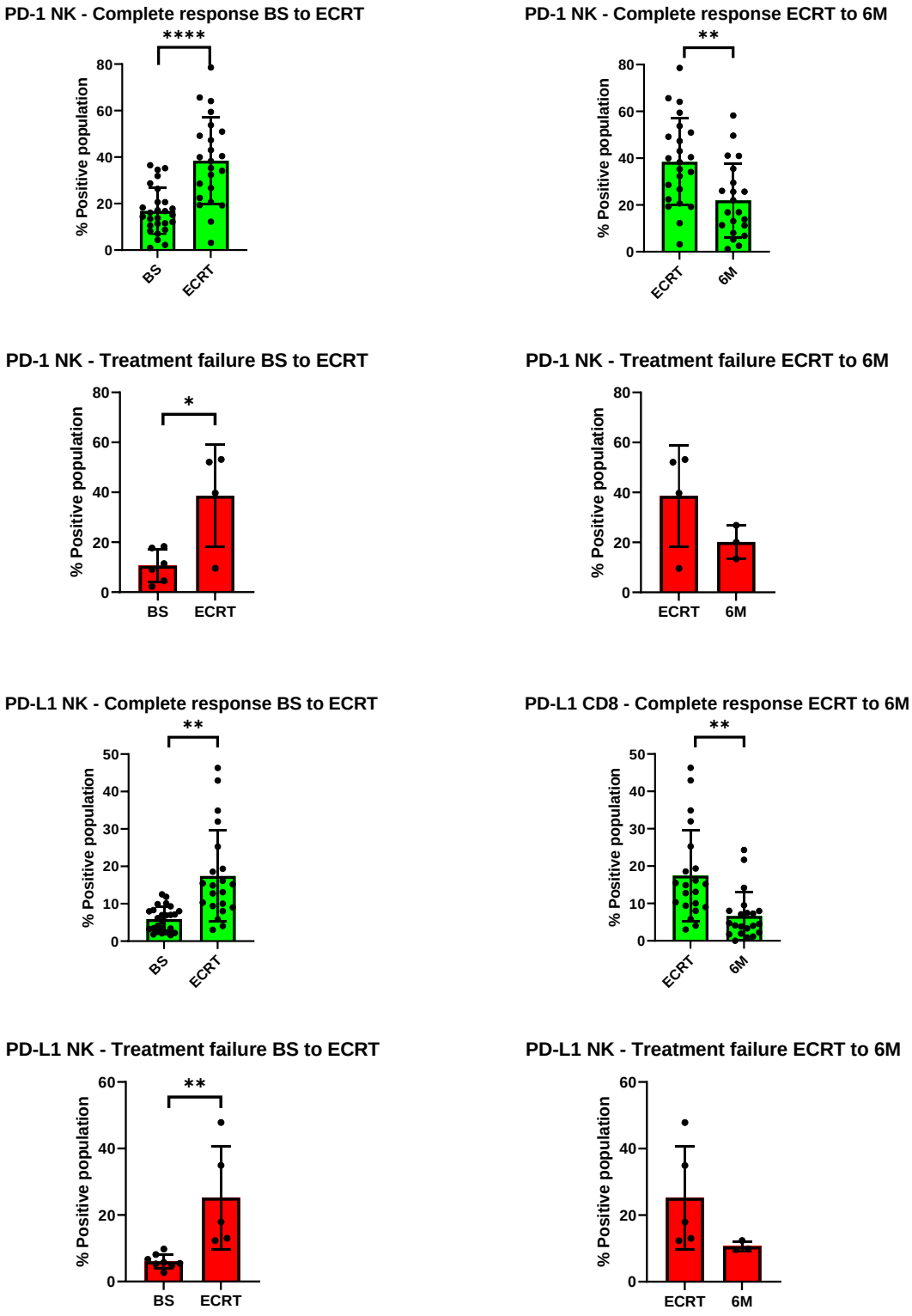


Figure 3.40 Changes in PD-1 and PD-L1 Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01, ✱✱✱✱P ≤ 0.0001

3.14 NK cell – exploratory markers

Figure 3.41, Figure 3.42, and Figure 1.38 show dynamic changes in CCR7, OX40, GITR, ICOS, CD40L, BTLA, CD69, CD137, LAG-3, TIM-3 and CD25 expression on NK cells in treatment failure and complete response groups, respectively. There were no statistically significant associations between groups.

Figure 3.44 to Figure 3.49 show changes in all markers from BS to ECRT and ECRT to 6M for complete response and treatment failure. As shown in Table 3.3, every marker had a statistically significant increase in expression from BS to ECRT in the complete response group, with all having a statistically significant decrease in expression from ECRT to 6M in the complete response groups, apart from CD137 which showed a trend in this direction. As shown in Table 3.4, similar trends were seen in the treatment failure groups.

Table 3.3 Mean % expression of exploratory markers on NK cells from BS to ECRT and ECRT to 6M in complete response.

Complete response BS to ECRT	Mean % Expression BS	Mean % Expression ECRT	Adjusted p-value
CCR7	6.74	18.30	0.0031
OX40	9.85	24.31	0.0004
GITR	10.21	23.53	0.0014
ICOS	4.80	15.20	0.0015
CD40L	5.93	17.18	≤ 0.0001
BTLA	6.34	9.85	0.0077
CD69	20.04	31.74	0.0122
CD137	9.84	18.35	0.0076
LAG-3	2.12	4.43	0.0094
TIM-3	24.85	33.54	0.0349
CD25	8.43	19.01	0.0023
Complete response ECRT to 6M	Mean % Expression ECRT	Mean % Expression 6M	Adjusted p-value
CCR7	18.30	6.55	0.0023
OX40	24.31	13.13	0.0102
GITR	23.53	12.65	0.0093
ICOS	15.20	4.51	0.0025
CD40L	17.18	9.12	0.0093
BTLA	9.85	7.33	0.0438
CD69	31.74	18.38	0.0093
CD137	18.35	13.16	0.0741
LAG-3	4.43	1.96	0.0093
TIM-3	33.54	24.76	0.0349
CD25	19.01	10.03	0.0093

Table 3.4 Mean % expression of exploratory markers on NK cells from BS to ECRT and ECRT to 6M in treatment failure.

Treatment failure BS to ECRT	Mean % Expression BS	Mean % Expression ECRT	Adjusted p-value
CCR7	4.47	21.17	0.0102
OX40	4.87	25.86	0.0093
GITR	9.97	21.93	0.0863
ICOS	4.23	23.57	0.0122
CD40L	9.04	21.14	0.1260
BTLA	4.25	8.19	0.0507
CD69	16.04	43.48	0.0147
CD137	5.97	11.84	0.0771
LAG-3	0.87	2.46	0.0721
TIM-3	20.81	31.52	0.1727
CD25	9.15	20.52	0.0749
Treatment failure ECRT to 6M	Mean % Expression ECRT	Mean % Expression 6M	Adjusted p-value
CCR7	21.17	8.28	0.1049
OX40	25.86	8.45	0.0721
GITR	21.93	14.03	0.2897
ICOS	23.57	6.01	0.0721
CD40L	21.14	4.94	0.0721
BTLA	8.19	4.62	0.1564
CD69	43.48	15.15	0.0462
CD137	11.84	9.34	0.3577
LAG-3	2.46	1.07	0.1727
TIM-3	31.52	15.62	0.1500
CD25	20.52	4.62	0.0665

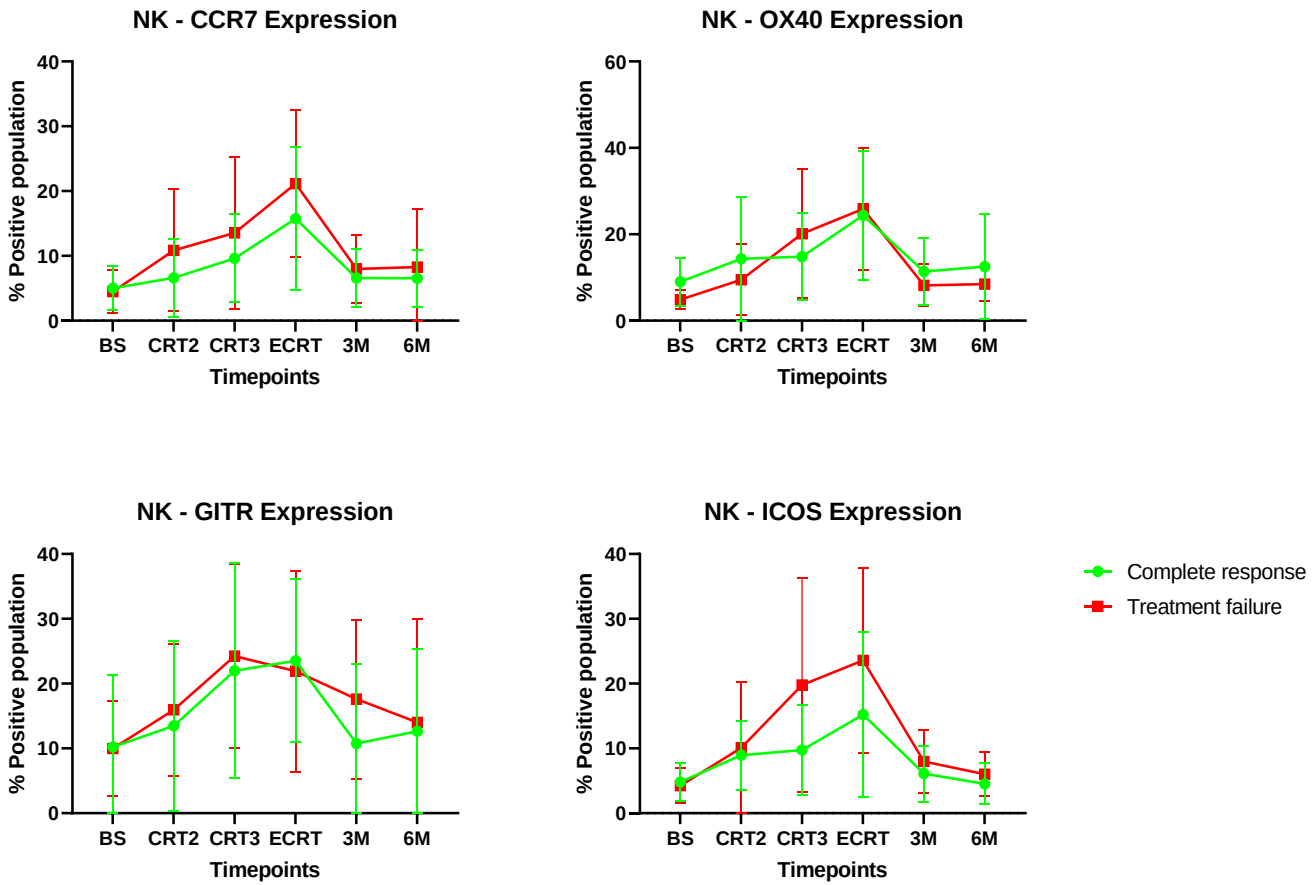


Figure 3.41 Dynamic changes in expression of CCR7, OX40, GITR and ICOS Expression on Natural Killer cells. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

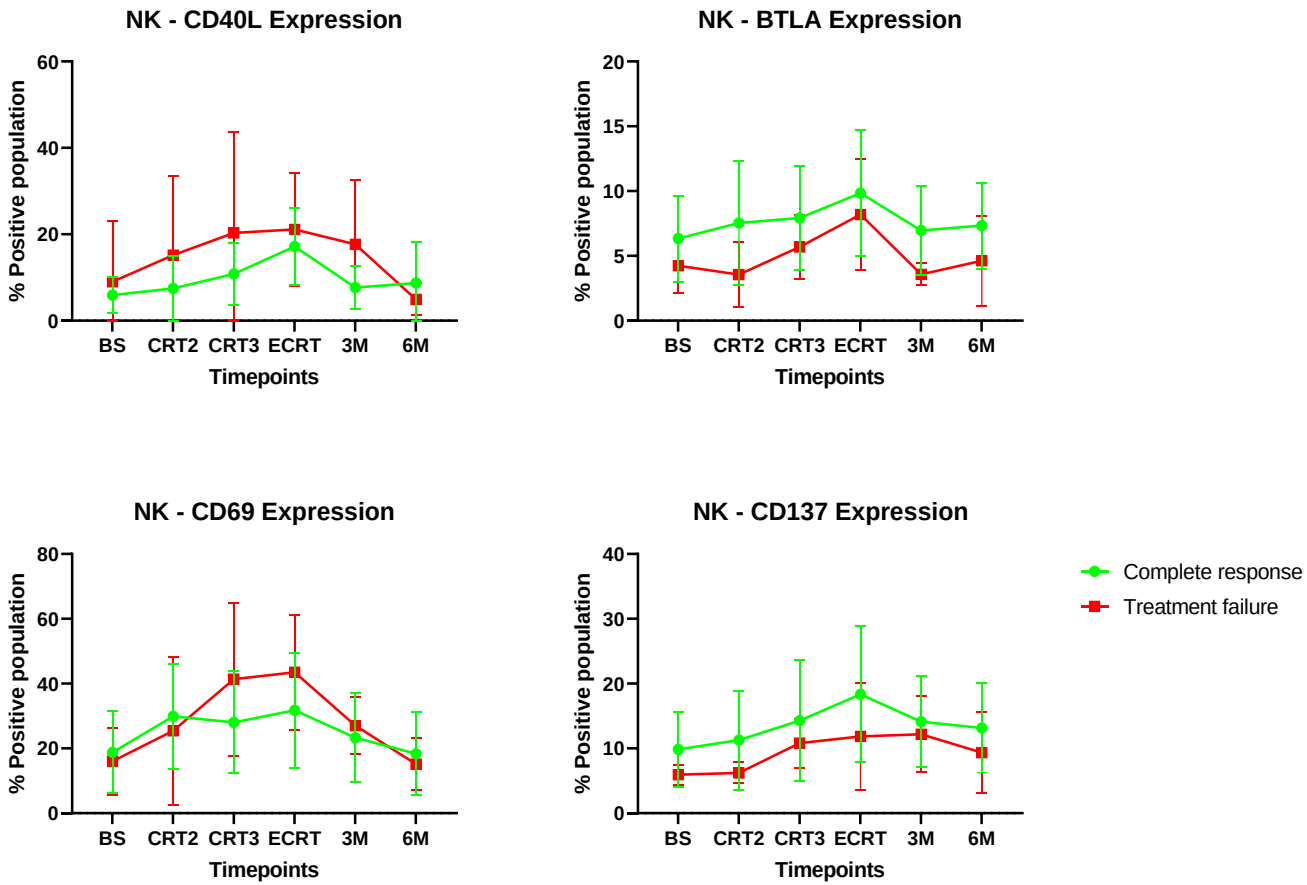


Figure 3.42 Dynamic changes in expression of CD40L, BTLA, CD69 and CD137 Expression on Natural Killer cells. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

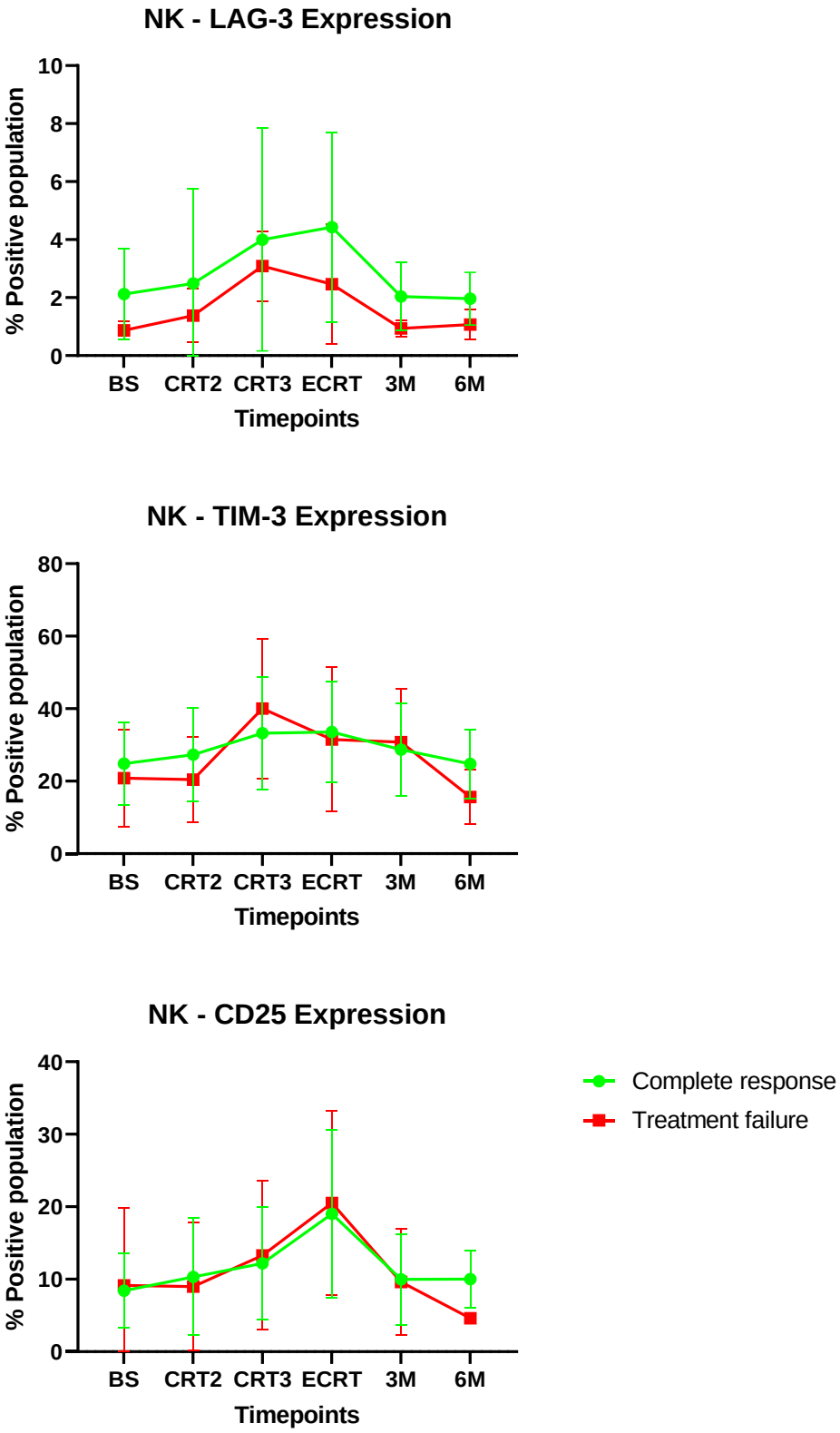


Figure 3.43 Dynamic changes in expression of LAG-3, TIM-3 and CD25 Expression on Natural Killer cells. Data is expressed as % positive of Natural Killer cells population +/- SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

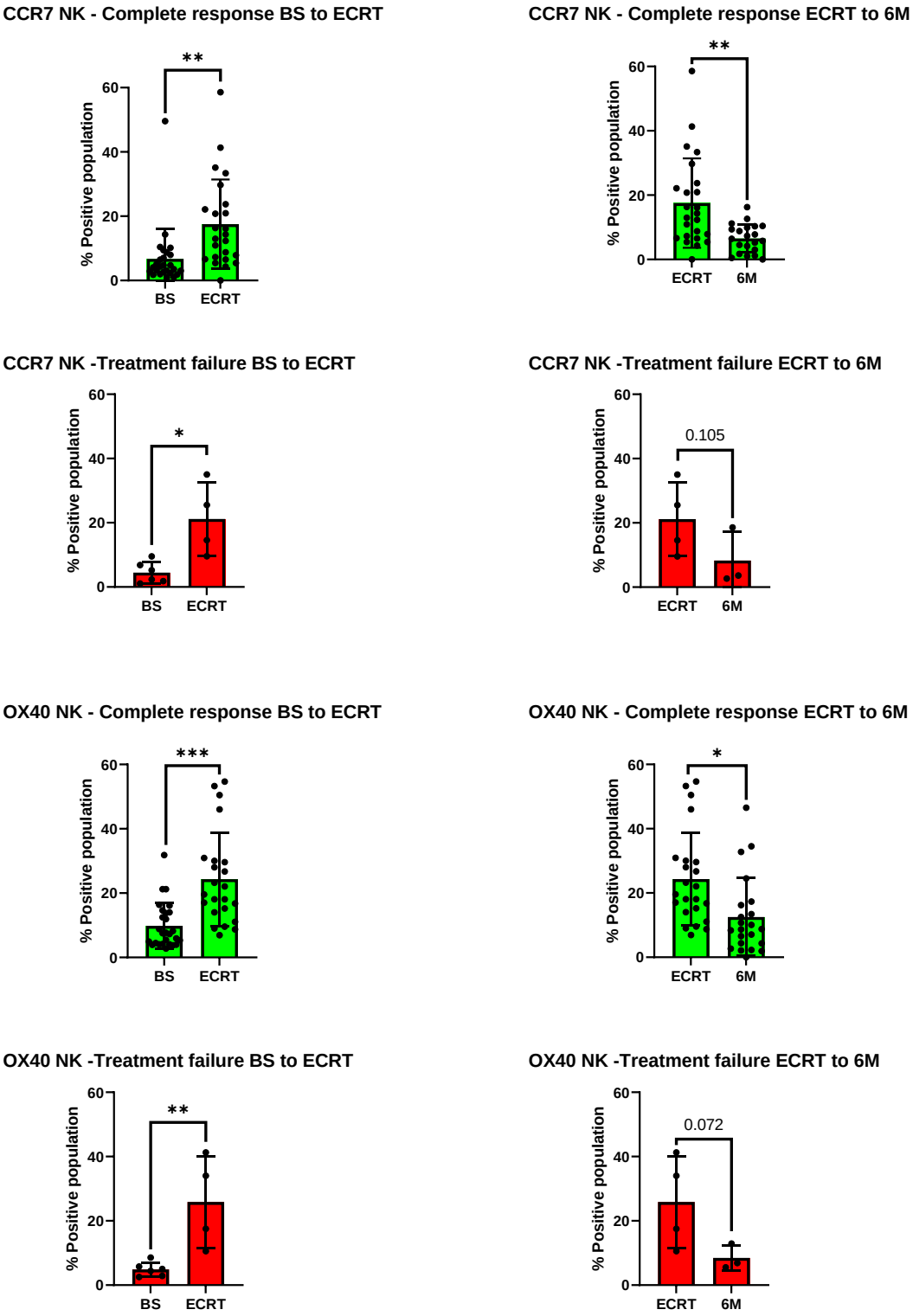


Figure 3.44 Changes in CCR7 and OX40 Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01, ✱✱✱P ≤ 0.001

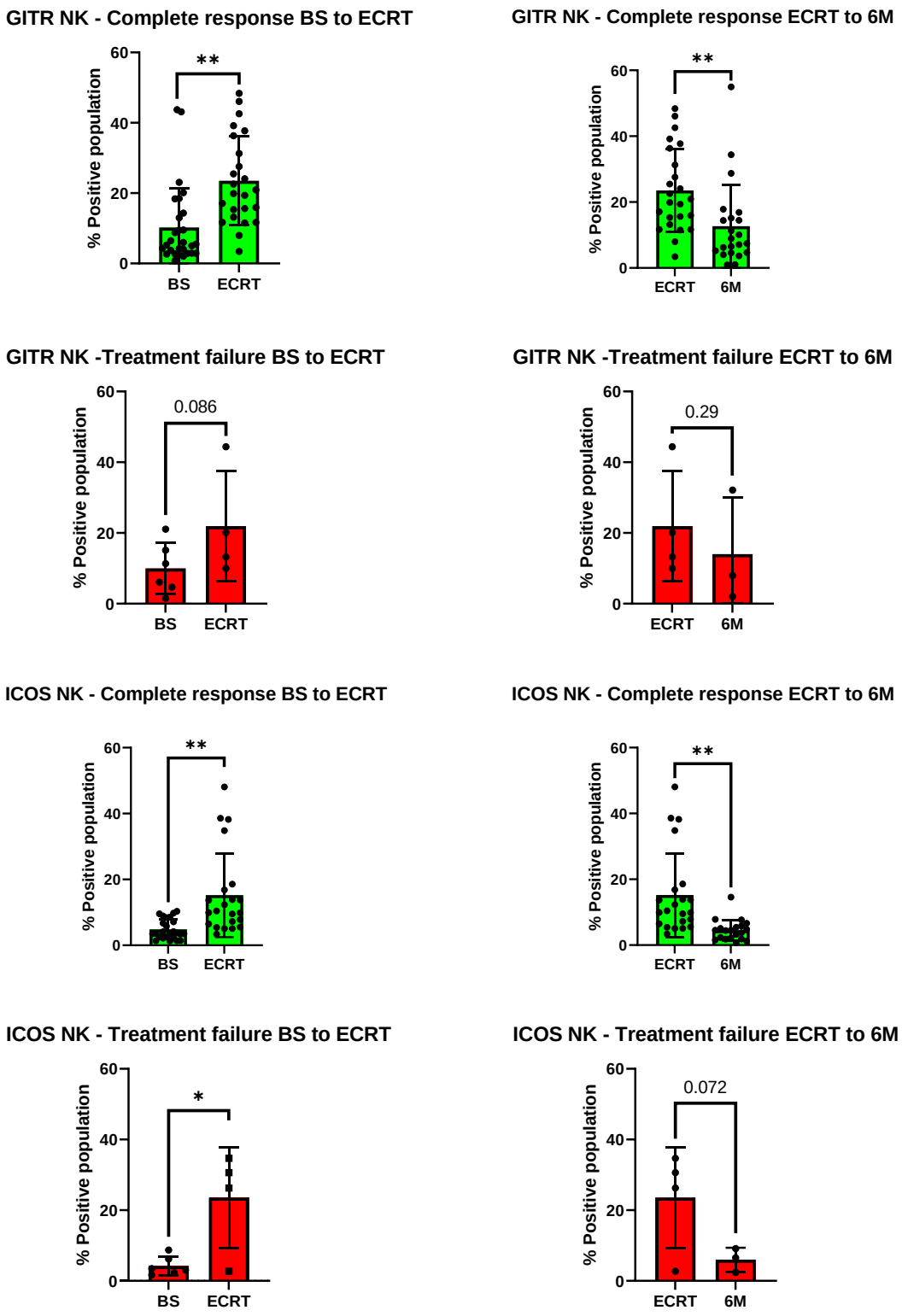


Figure 3.45 Changes in GITR and ICOS Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/- SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01

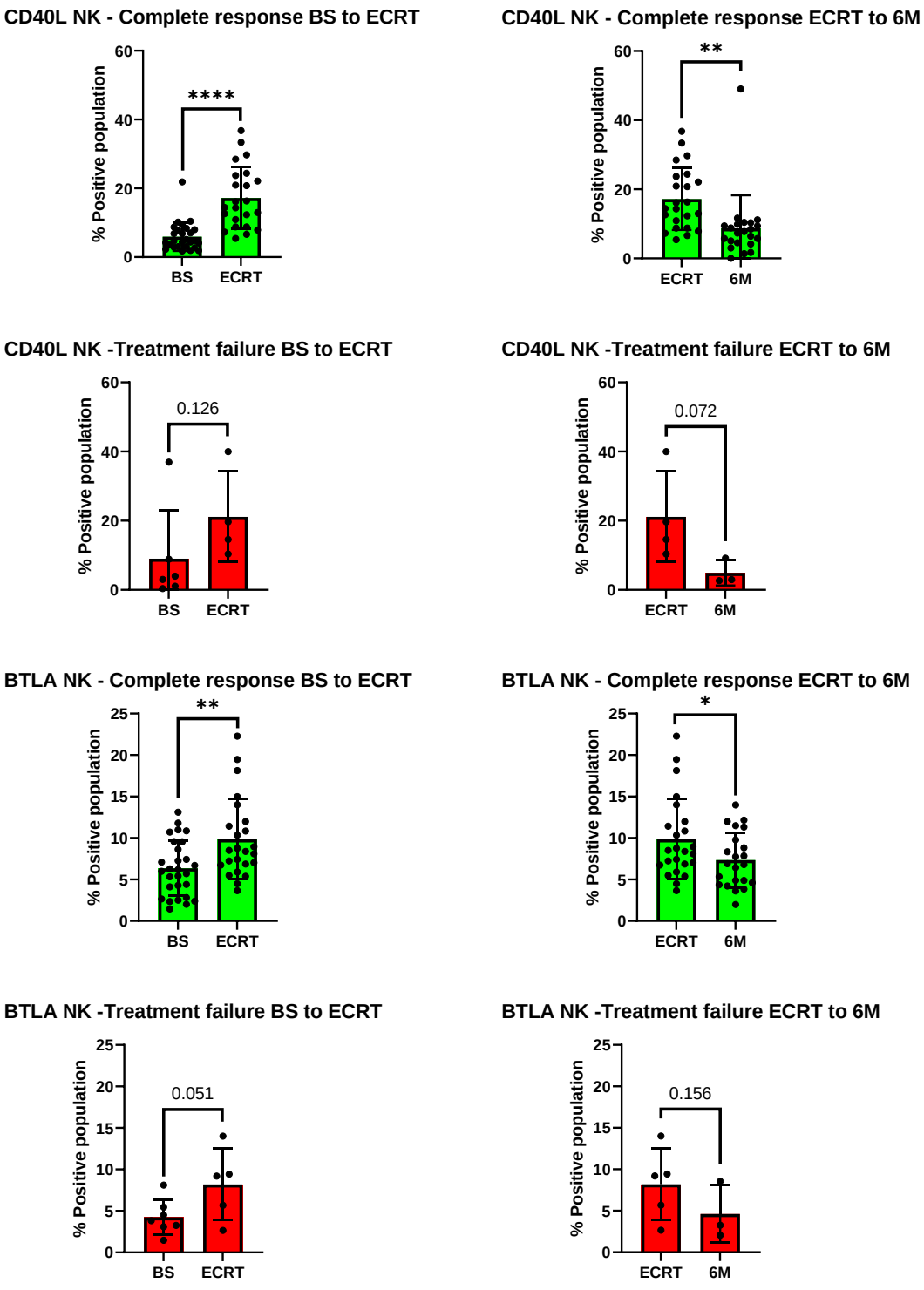


Figure 3.46 Changes in CD40L and BTLA Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01, ✱✱✱✱P ≤ 0.0001

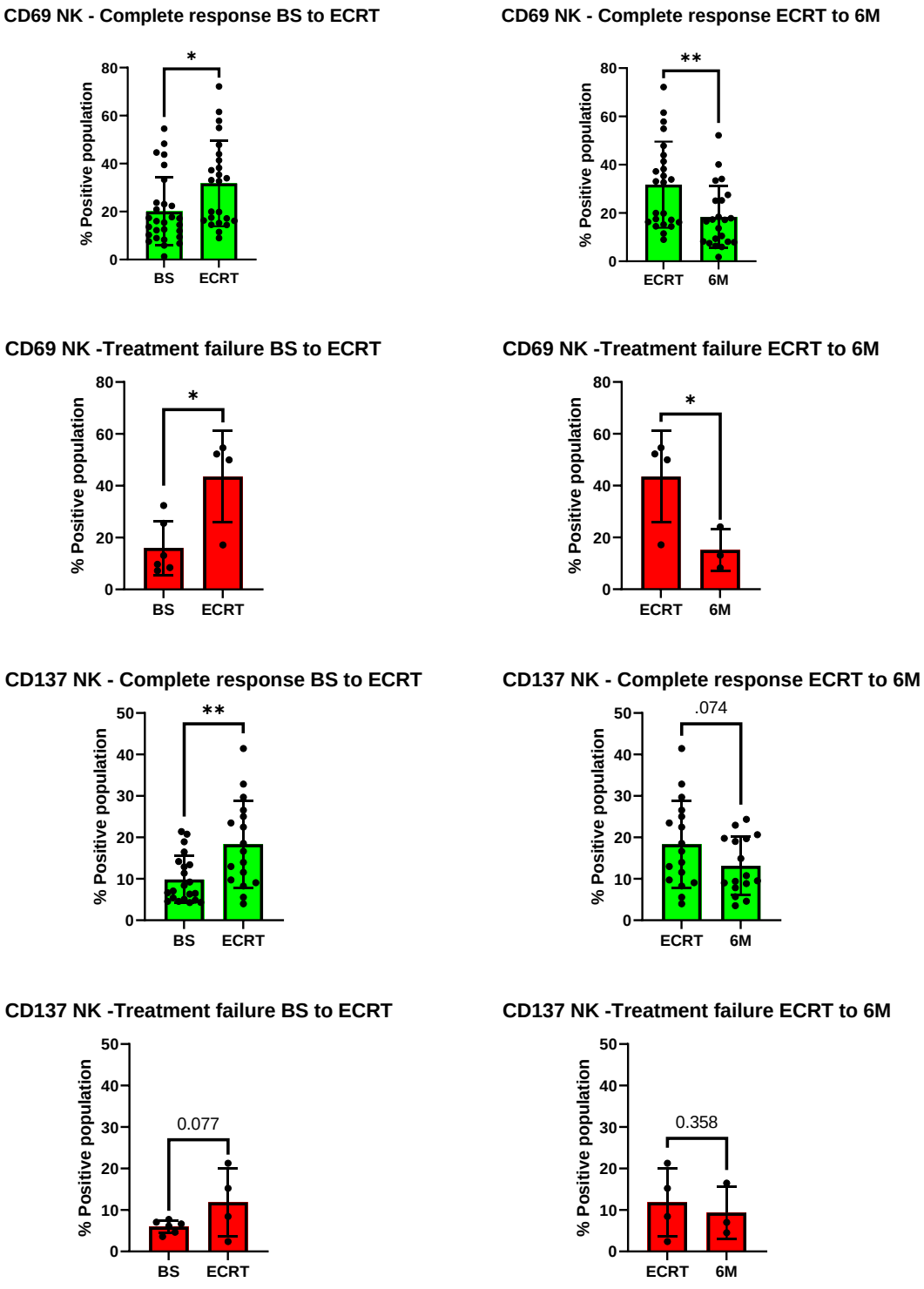


Figure 3.47 Changes in CD69 and CD137 Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01

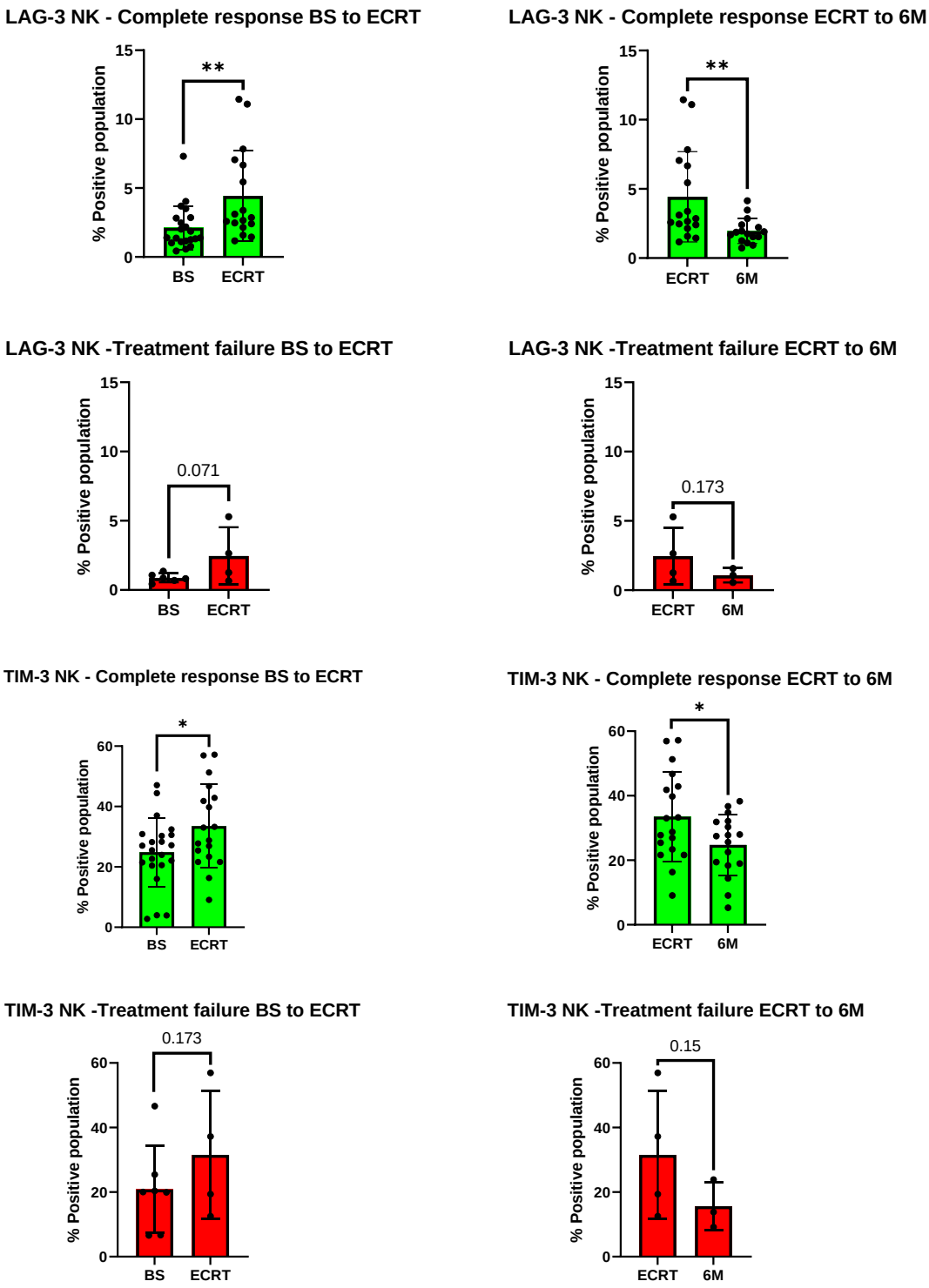


Figure 3.48 Changes in LAG-3 and TIM-3 Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01

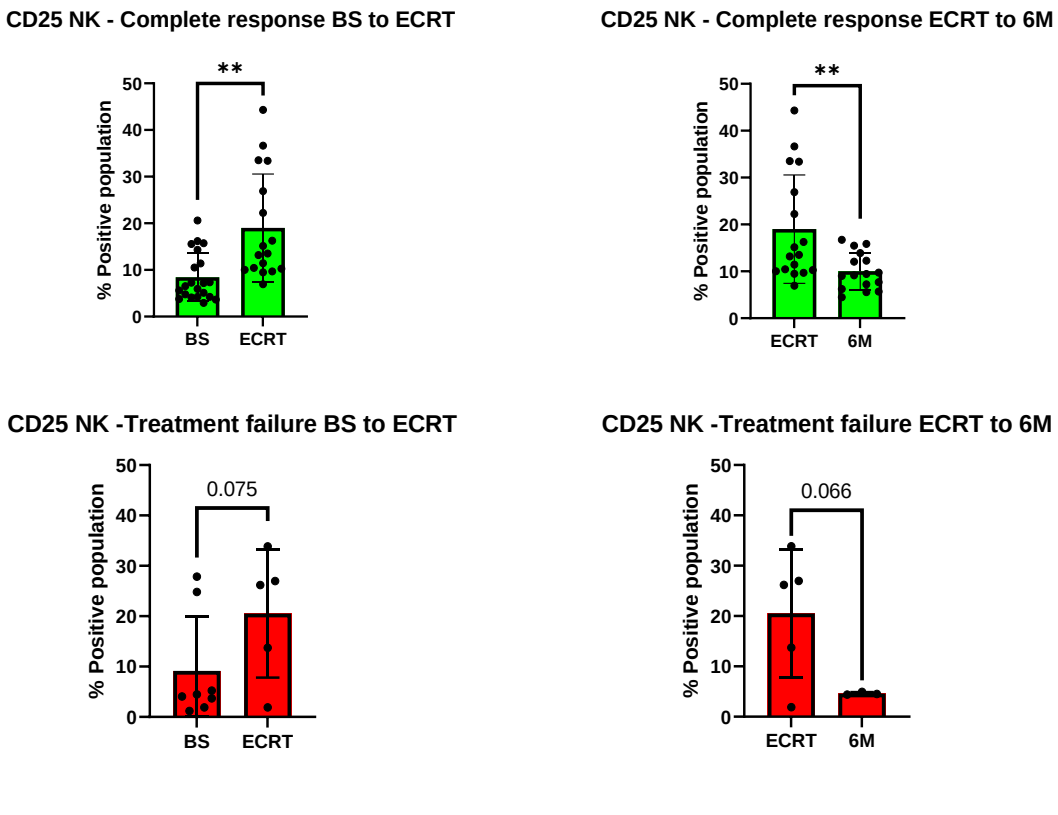
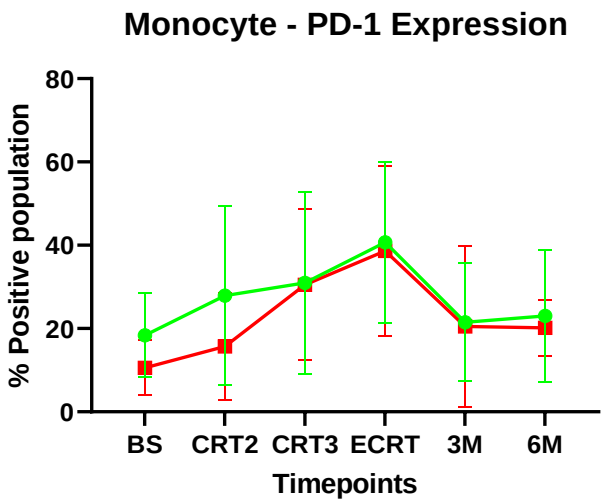


Figure 3.49 Changes in CD25 Expression on Natural Killer cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of Natural Killer cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱✱P ≤ 0.01

3.15 Monocyte – targeted markers

Figure 3.50 shows dynamic changes in PD-1 and PD-L1 expression on Monocytes in treatment failure and complete response. Figure 3.51 compares PD-1 and PD-L1 expression on monocytes at BS, ECRT, and 6M for complete response and treatment failure. There were no statistically significant differences between groups.

Figure 3.52 shows changes during and after treatment for these markers within groups. Within groups, PD-1 showed a statistically significant increase from baseline to ECRT in complete response (18.4 % PD-1 expression in complete response at BS to 40.69 % at ECRT, adjusted p-value ≤0.0001) and treatment failure (10.57 % PD-1 expression in treatment failure at BS to 38.63 % at ECRT, adjusted p-value 0.004). There was a statistically significant decrease from ECRT to 6M for PD-1 in the complete response group (40.69% PD-1 expression in complete response at ECRT to 23.03% at 6M, adjusted p-value 0.003), with a similar trend for treatment failure (38.63 % PD-1 expression in treatment failure at BS to 20.14 % at ECRT, adjusted p-value 0.051). Similar results were seen for PD-L1, with a statistically significant increase from baseline to ECRT in complete response (11.4 % PD-L1 expression in complete response at BS to 23.49 % at ECRT, adjusted p-value 0.004) and treatment failure (6.1 % PD-L1 expression in treatment failure at BS to 25.2 % at ECRT, adjusted p-value 0.003), followed by a statistically significant decrease from ECRT to 6M for complete response (23.49 % PD-L1 expression in complete response at ECRT to 9.35 % at 6M, adjusted p-value 0.004) and treatment failure (25.2 % PD-L1 expression in treatment failure at ECRT to 10.62 % at 6M, adjusted p-value 0.051)



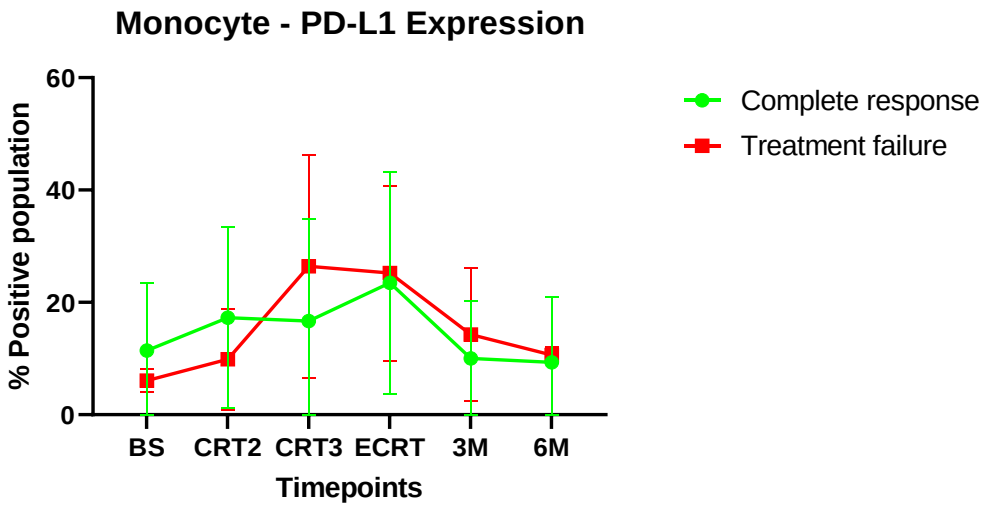


Figure 3.50 Dynamic changes in expression of PD-1 and PD-L1 Expression on monocytes. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

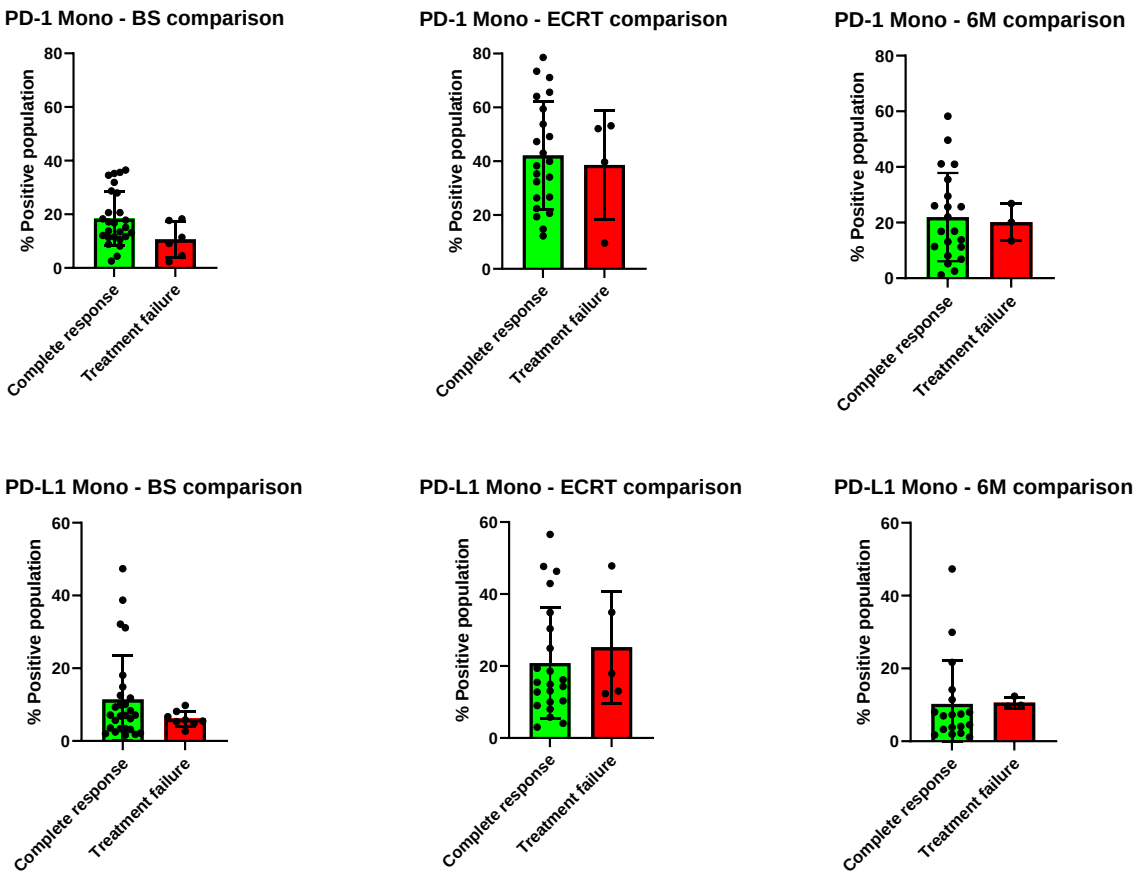


Figure 3.51 Comparison between complete response and treatment failure groups for PD-1 and PD-L1 on monocytes. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

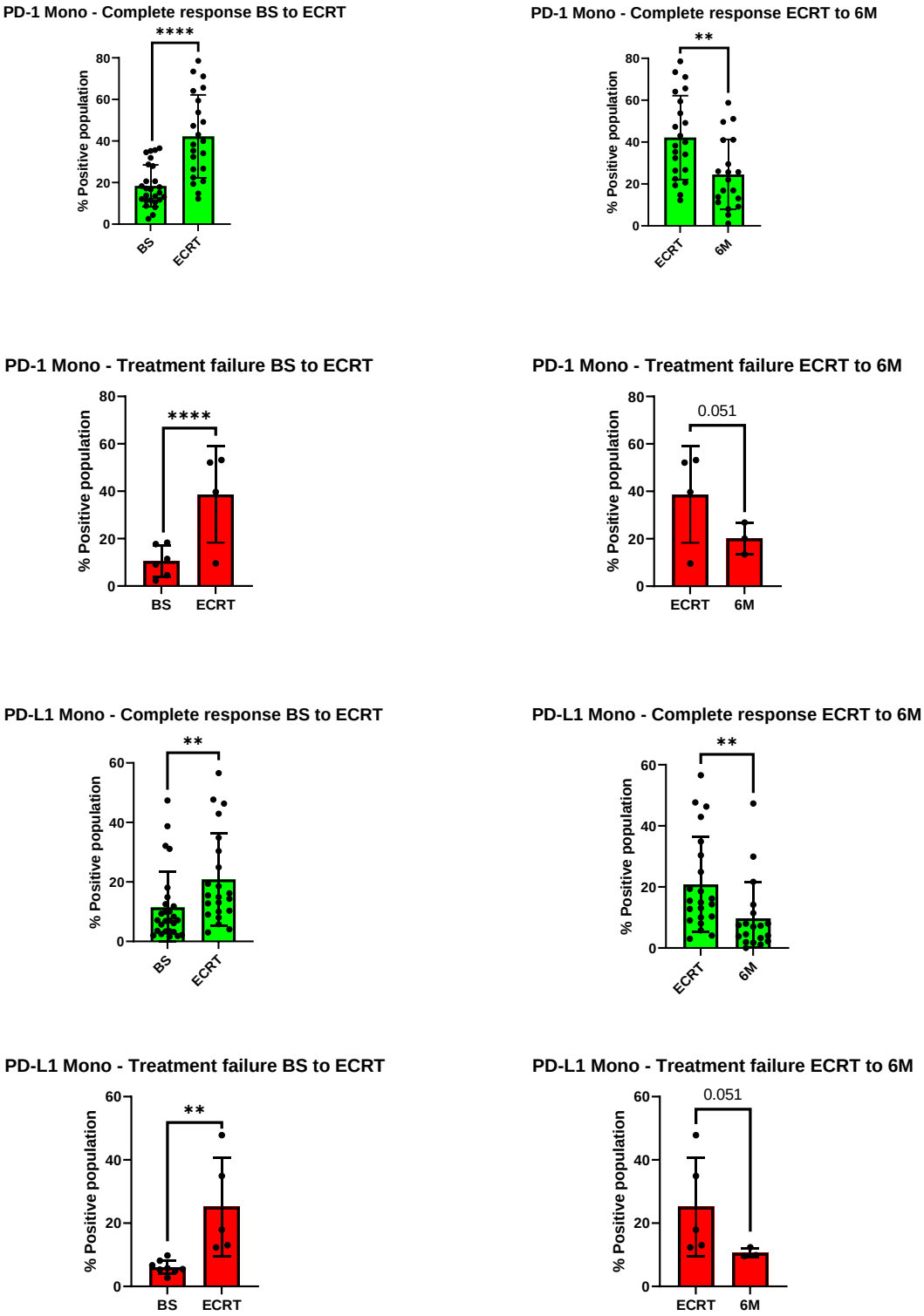


Figure 3.52 Changes in PD-1 and PD-L1 Expression on monocytes from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01, ✱✱✱✱P ≤ 0.0001

3.16 Monocyte – exploratory markers

Figure 3.53 to Figure show dynamic changes CD16, HLA-DR, CD86, CD80, ICOS-L, HVEM, CD40, CD69, Gal-9, OX40L, HLA-ABC, CD137L, CD70, CD155 and CD112 in treatment failure and complete response groups.

Non-parametric tests were used for HLA-DR, CD112 and CD155. Figure 3.57 shows the difference between CD112 and CD155 expression on monocytes at baseline, ECRT and 6M. There was a statistically significant higher median expression of CD112 at baseline in patients with treatment failure (95.2 % CD112 expression at baseline in treatment failure versus 72.8 % in complete response, adjusted p-value 0.024). There was a trend for this difference to be maintained to ECRT (median 99.64 % CD122 expression at ECRT in treatment failure versus 82.17 % in complete response, adjusted p-value 0.119) and 6M (median 93.65 % CD112 expression at 6M in treatment failure versus 68.37 % in complete response, adjusted p-value 0.119). No difference was seen in CD155. Data for CD155 is shown given its relevance as the most specific ligand for TIGIT. There were no statistically significant differences within groups.

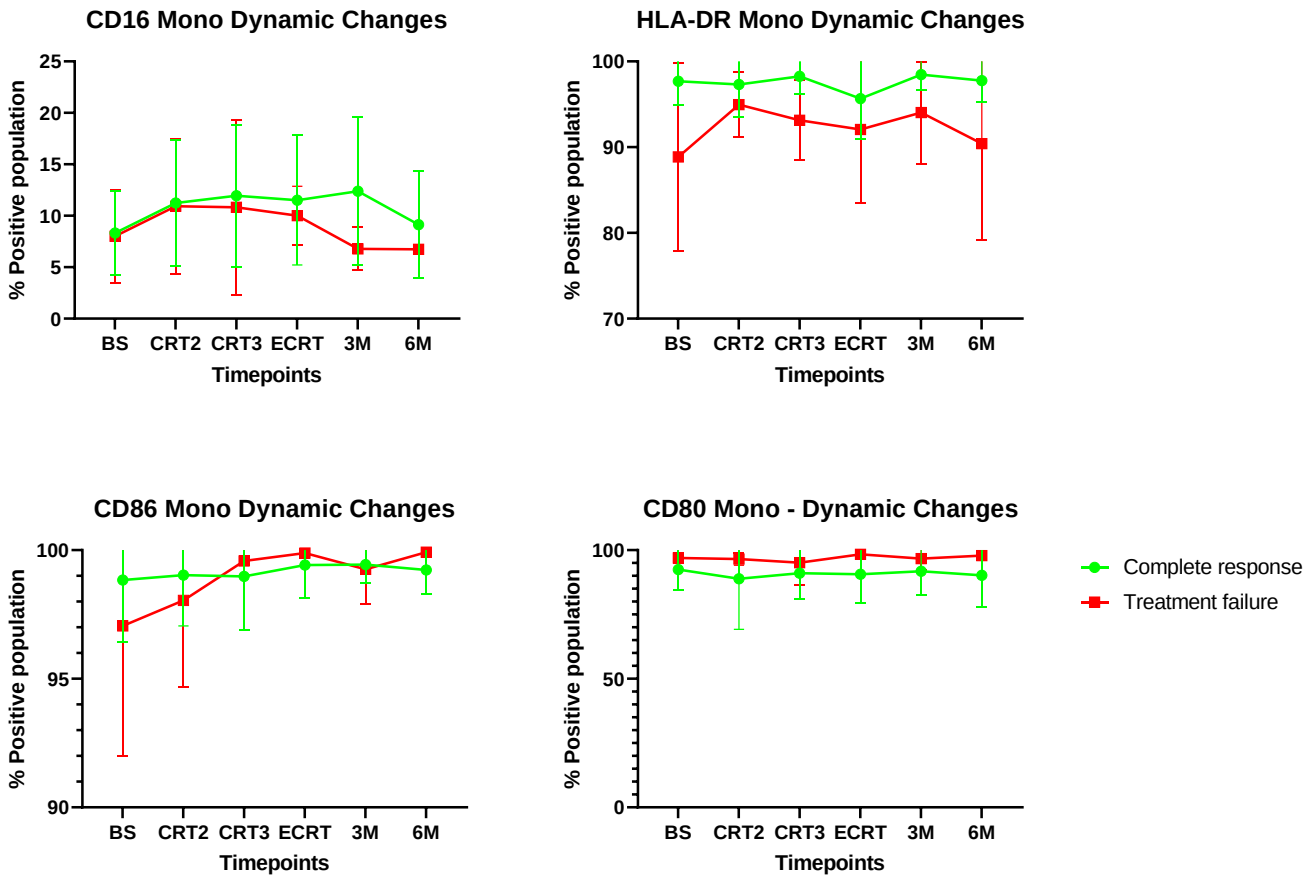


Figure 3.53 Dynamic changes in expression of CD16, HLA-DR, CD86 and CD80 Expression on monocytes. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

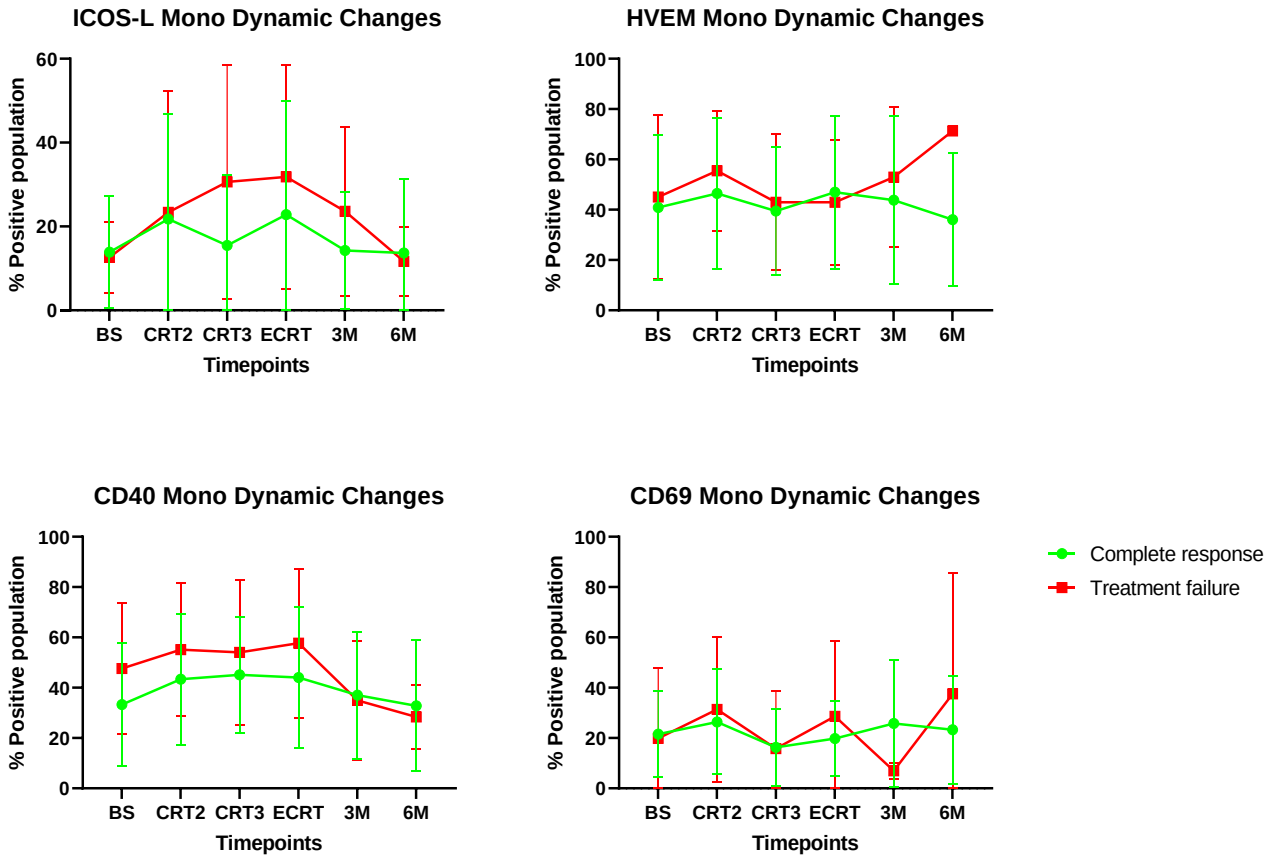


Figure 3.54 Dynamic changes in expression of ICOS-L, HVEM, CD40 and CD69 Expression on monocytes. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

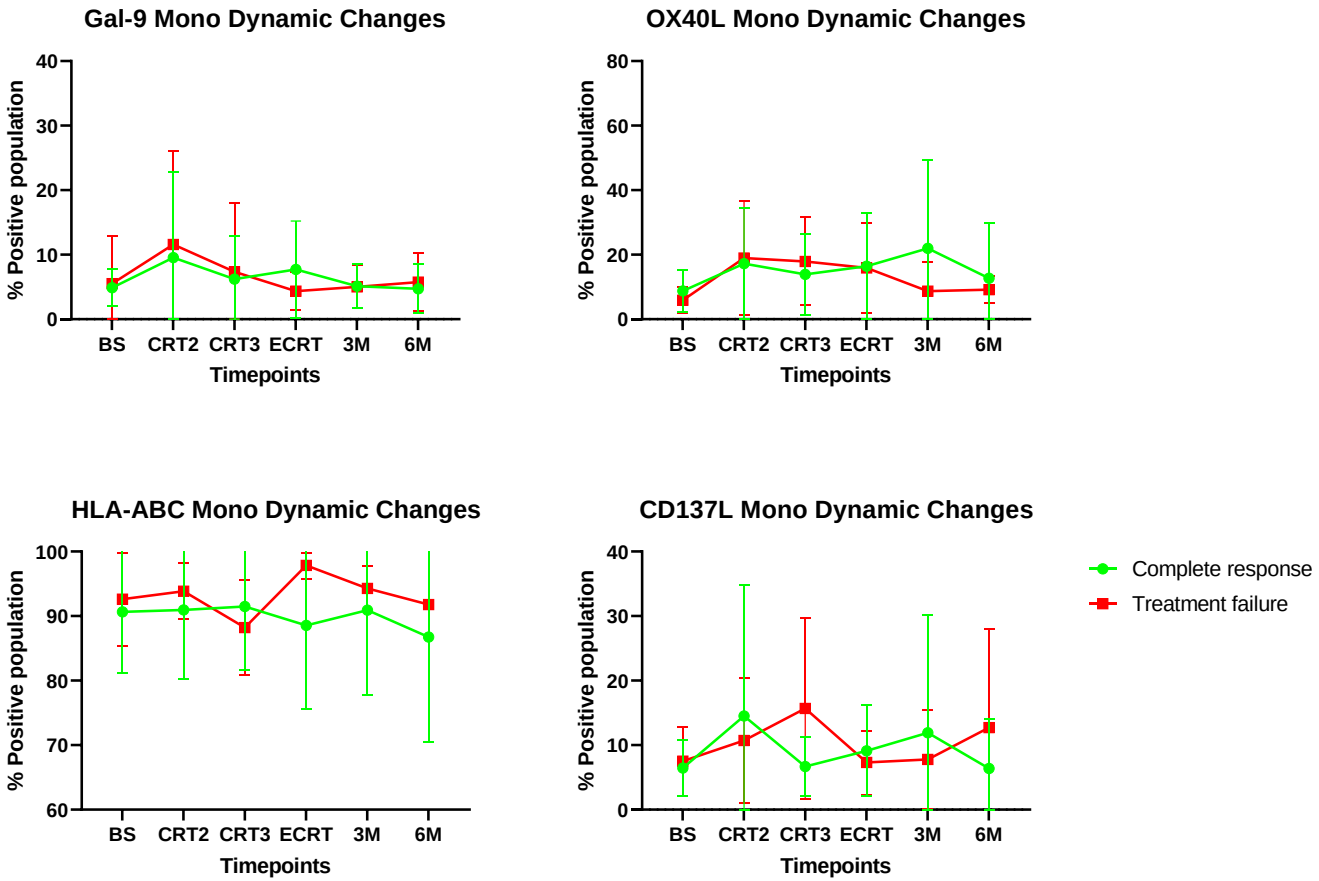


Figure 3.55 Dynamic changes in expression of Gal-9, OX40L, HLA-ABC and CD137L Expression on monocytes. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

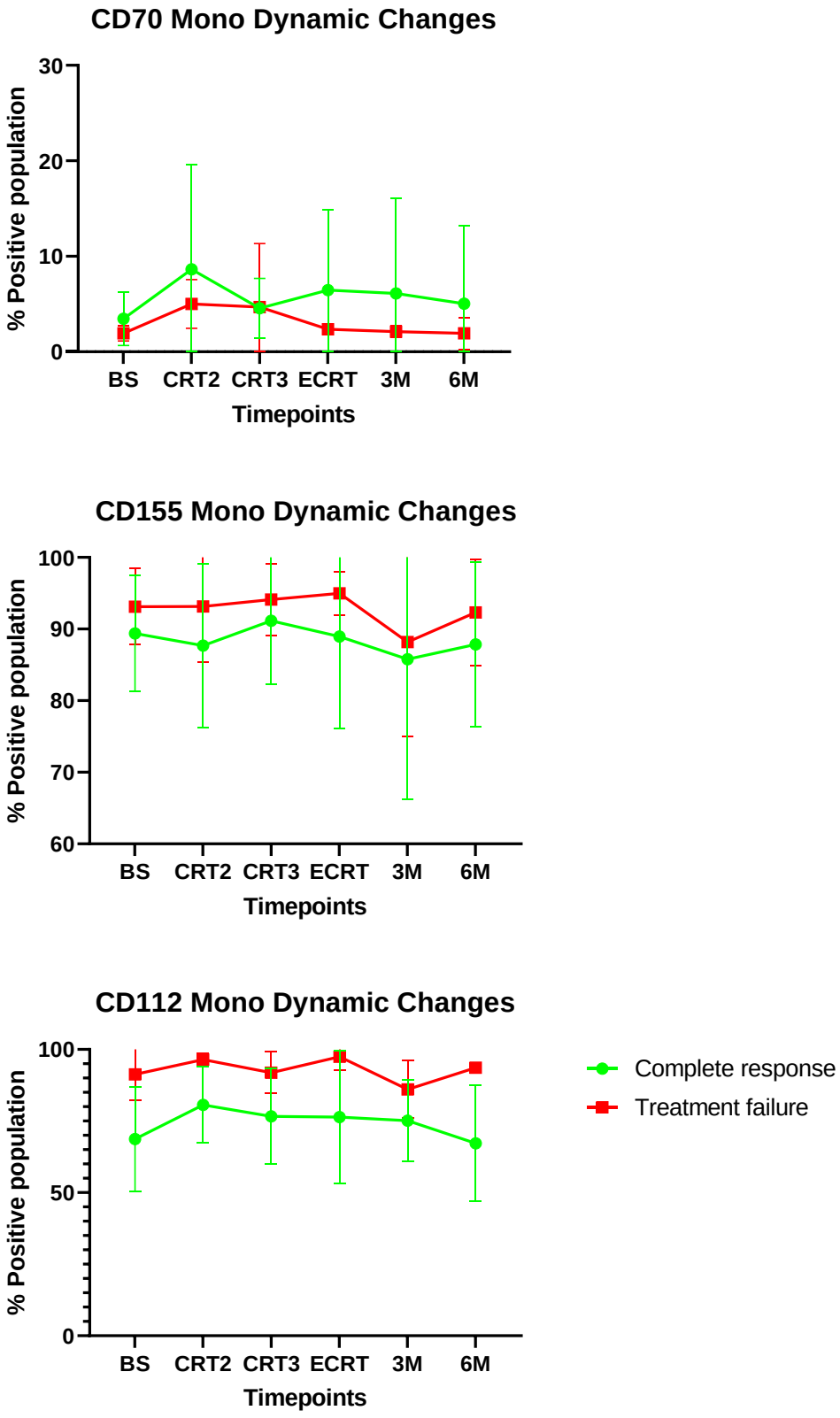


Figure 3.56 Dynamic changes in expression of CD70, CD155 and CD112 Expression on monocytes. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

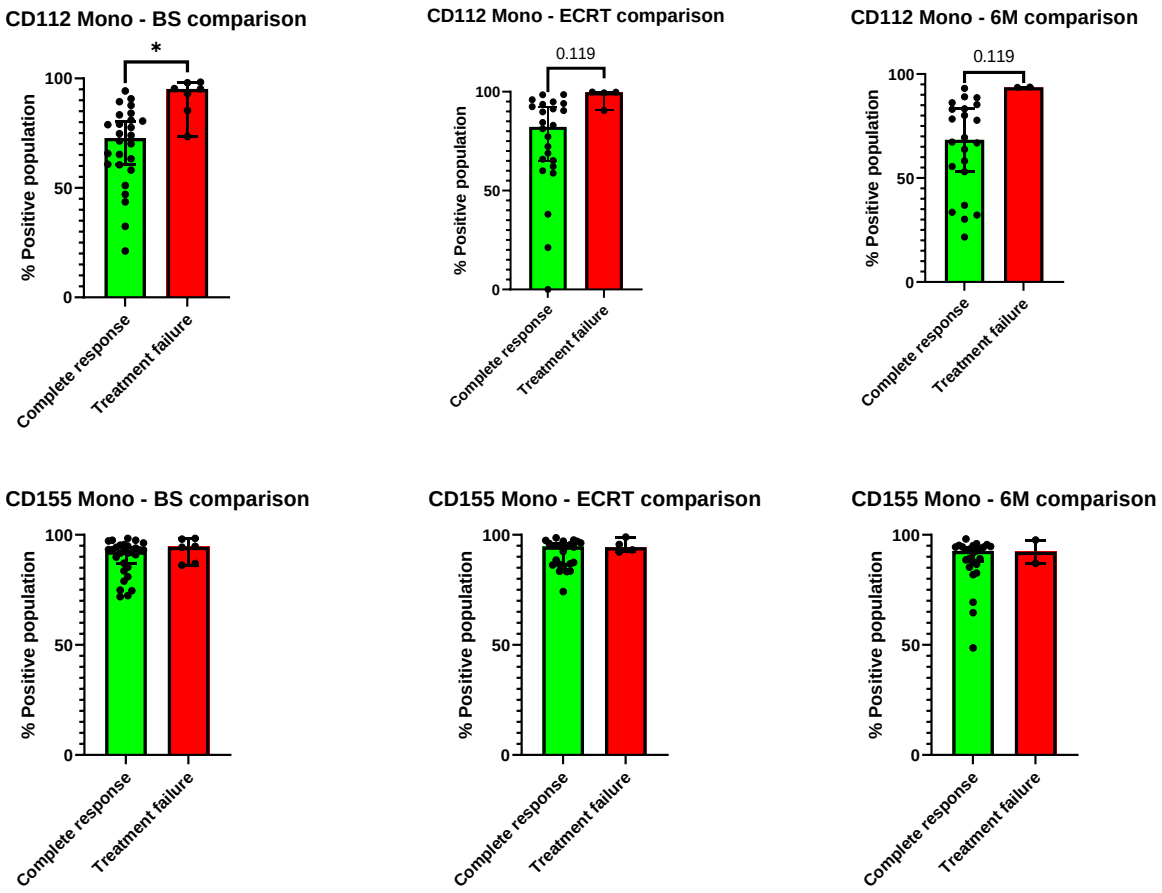


Figure 3.57 Comparison between complete response and treatment failure groups for CD112 and CD155 on monocytes. Data is expressed as % positive of monocyte population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05

3.17 B-cells – targeted markers

Figure 3.58 shows dynamic PD-1 and PD-L1 expression changes on B-cells in treatment failure and complete response groups. Figure 3.59 compares PD-1 and PD-L1 expression on B-cells at BS, ECRT and 6M for complete response and treatment failure. There were no statistically significant differences between groups.

Figure 3.60 shows changes during and after treatment for these markers within groups. Within groups, PD-1 showed a statistically significant increase from baseline to ECRT in complete response (22.8 % PD-1 expression in complete response at BS to 37.98 % at ECRT, adjusted p-value 0.0002) and treatment failure (19.02 % PD-1 expression in treatment failure at BS to 48.16 % at ECRT, adjusted p-value 0.0011) There was a statistically significant decrease from ECRT to 6M for PD-1 in the complete response group (37.98 % PD-1 expression in complete response at ECRT to 22.9 % at 6M, adjusted p-value 0.0002) and treatment failure (48.16 % PD-1 expression at ECRT to 19.12 % at 6M, adjusted p-value 0.02). Similar results were seen for PD-L1, with a statistically significant increase from baseline to ECRT in complete response (5.05 % PD-L1 expression in complete response at BS to 13.41 % at ECTY, adjusted p-value ≤ 0.0001) and treatment failure (4.9 % PD-L1 expression in treatment failure at BS to 15.13 % at ECRT, adjusted p-value ≤ 0.0001) This was followed by statistically significant decrease from ECRT to 6M for complete response (13.41 % PD-L1 expression in complete response at ECRT to 6.04 % at 6M, adjusted p-value 0.0002) and treatment failure (15.13 % PD-L1 expression in treatment failure at ECRT to 7.5 % at 6M, adjusted p-value 0.02)

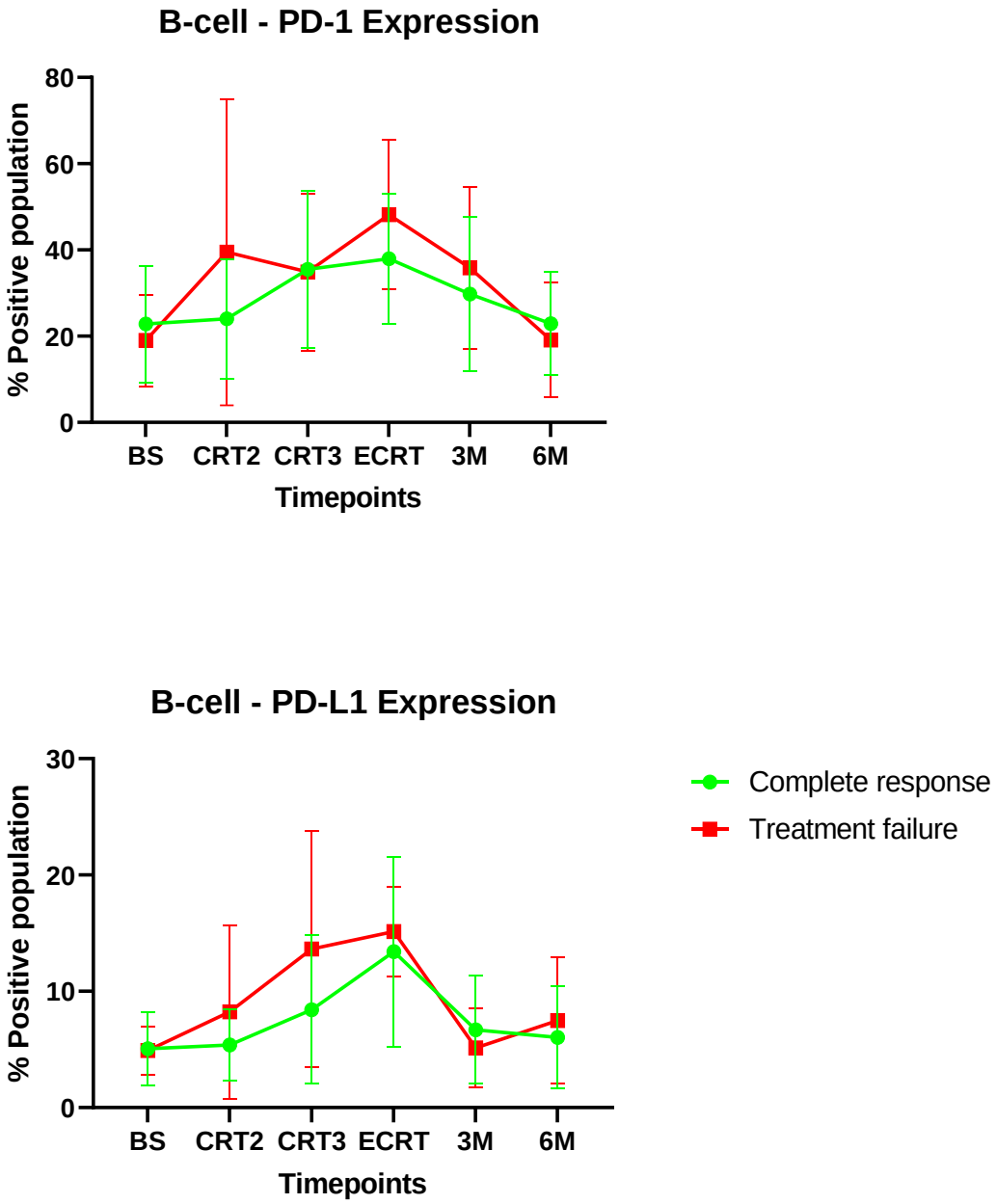


Figure 3.58 Dynamic changes in expression of PD-1 and PD-L1 Expression on B-cells. Data is expressed as % positive of B-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

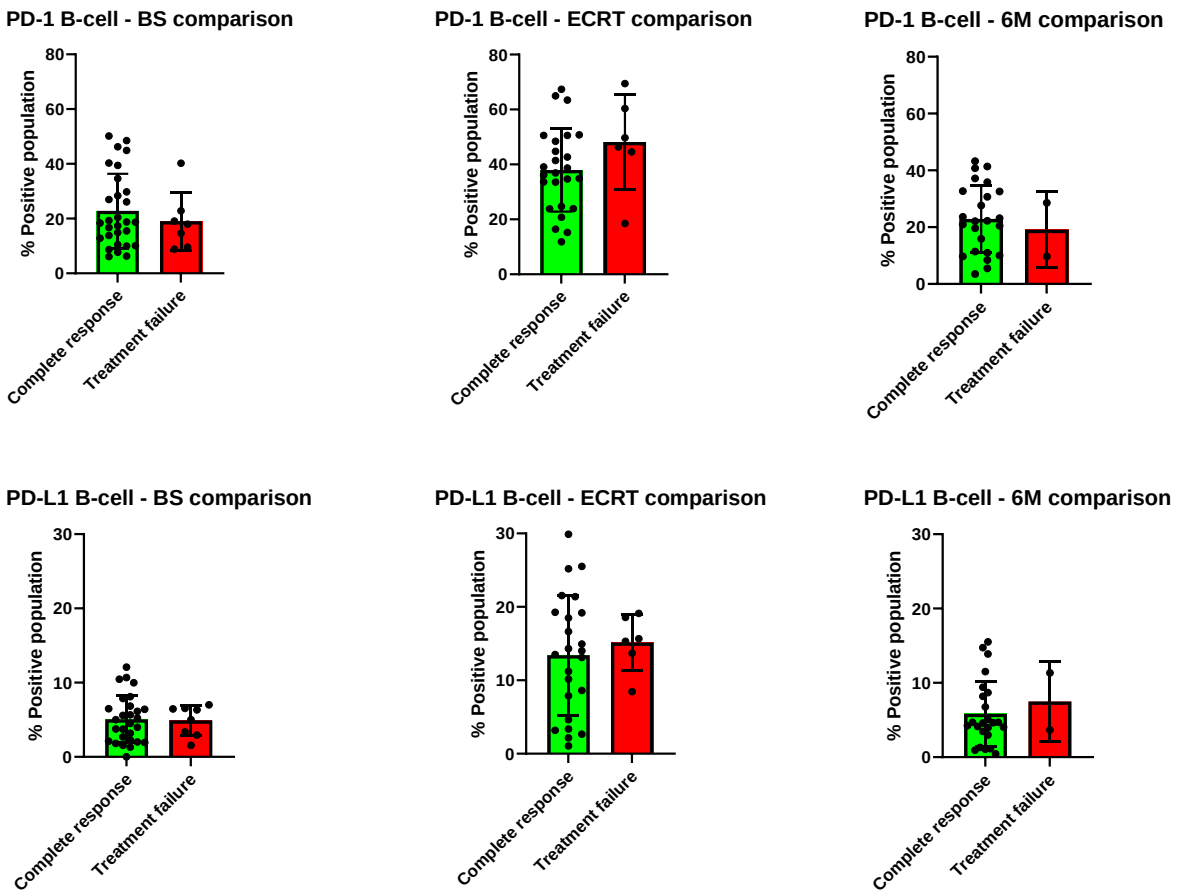


Figure 3.59 Comparison between complete response and treatment failure groups for PD-1 and PD-L1 on B-cells. Data is expressed as % positive of B-cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

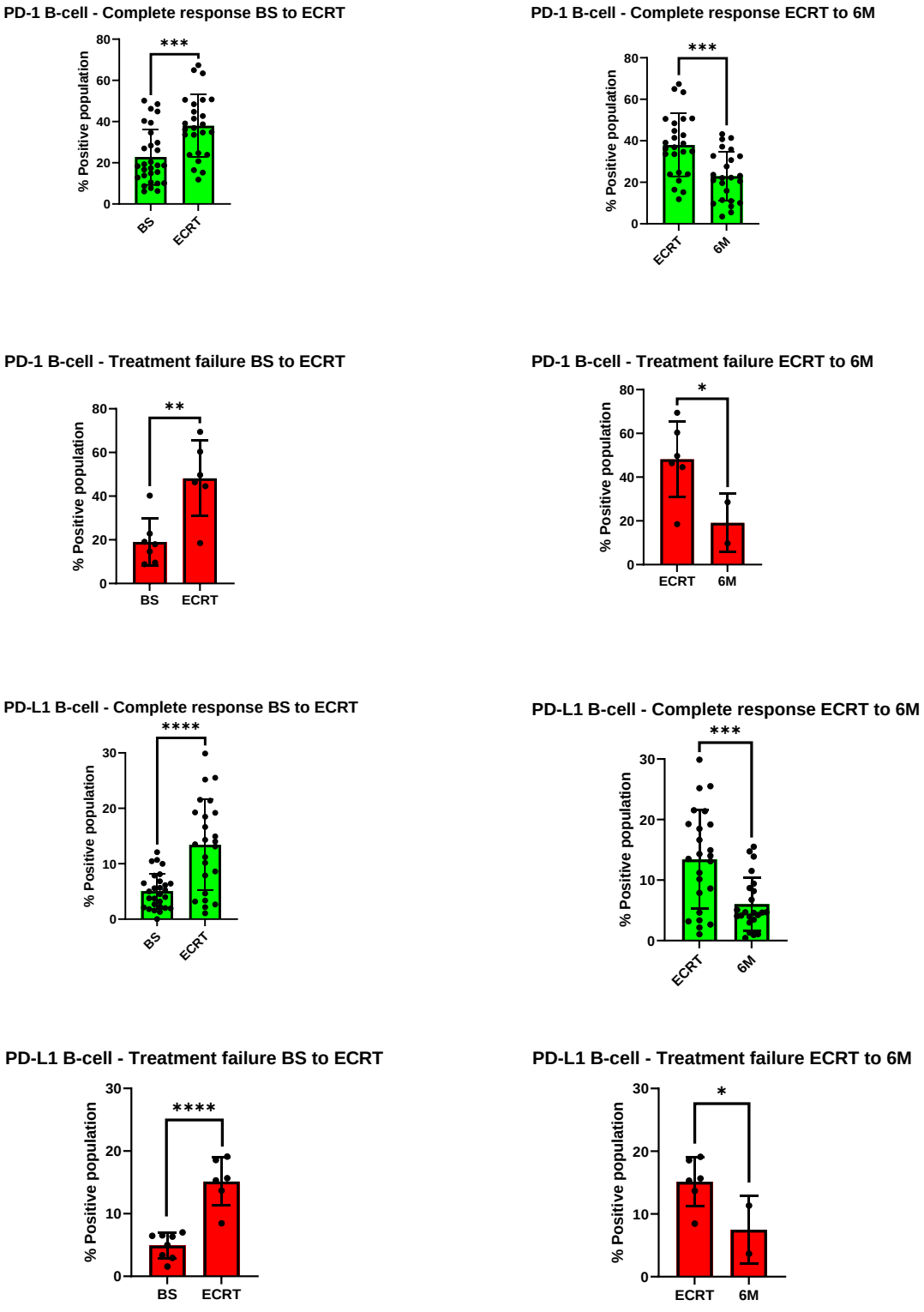


Figure 3.60 Changes in PD-1 and PD-L1 Expression on B-cells from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as % positive of B-cells population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent. ✱P ≤ 0.05, ✱✱P ≤ 0.01, ✱✱✱P ≤ 0.001, ✱✱✱✱P ≤ 0.0001

3.18 B-cells – exploratory markers

Figure 3.61 to Figure 3.64 show dynamic changes CD16, HLA-DR, CD86, CD80, ICOS-L, HVEM, CD40, CD69, Gal-9, OX40L, HLA-ABC, CD137L, CD70, CD155 and CD112 in treatment failure and complete response groups. As with the expected B-cell markers, lack of samples at 6M limits comparisons. Non-parametric tests were performed on HLA-DR, CD40, CD69, CD70 and CD112. There were no statistically significant differences between groups. Figure 3.65 shows comparisons for CD112 and CD155, given their role as ligands for TIGIT.

As shown in Figure 3.66, in the complete response group there was a statistically significant increase in CD86 from BS to ECRT (median 8.72 % CD86 expression in complete response at BS to 20.73 % at ECRT, adjusted p-value 0.0002) and CD112 (median 3.89 % CD122 expression in complete response at BS to 8.24 % at ECRT, adjusted p-value 0.0128). In the complete response group, there was a decrease in CD40 from BS to ECRT (median 82.42 % CD40 expression in complete response at BS to 58.64 % at ECRT, adjusted p-value 0.0025). In the complete response group, there was a statistically significant decrease from ECRT to 6M in Gal-9 (median 20.24 % Gal-9 expression in complete response at ECRT to 6.45% at 6M, adjusted p-value 0.0025) and a decrease in CD86 from ECRT to 6M(median 20.73 % CD86 expression in complete response at ECRT to 8.91 % at 6M, adjusted p-value 0.0025).

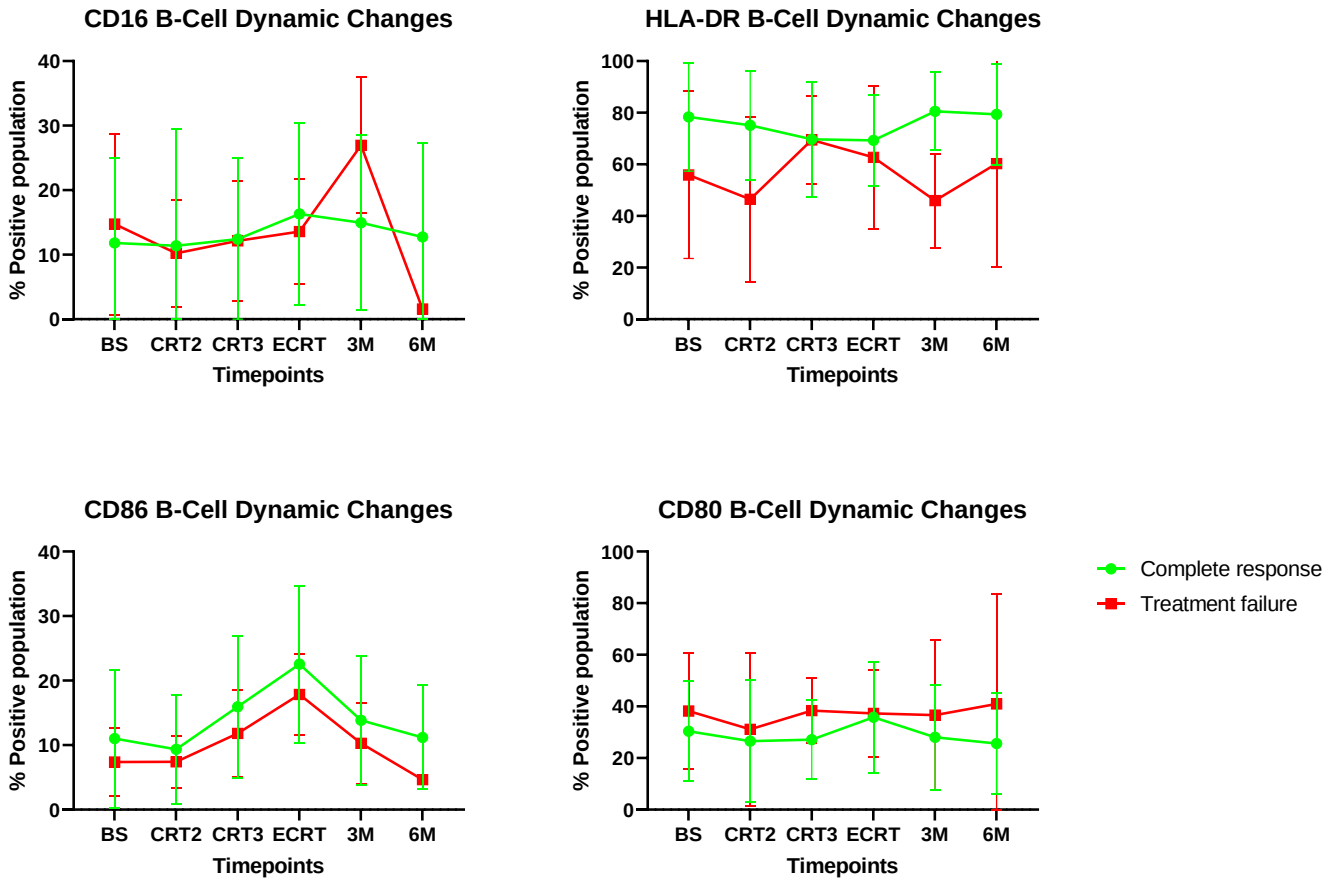


Figure 3.61 Dynamic changes in expression of CD16, HLA-DR, CD86 and CD80 Expression on B-cells. Data is expressed as % positive of B-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

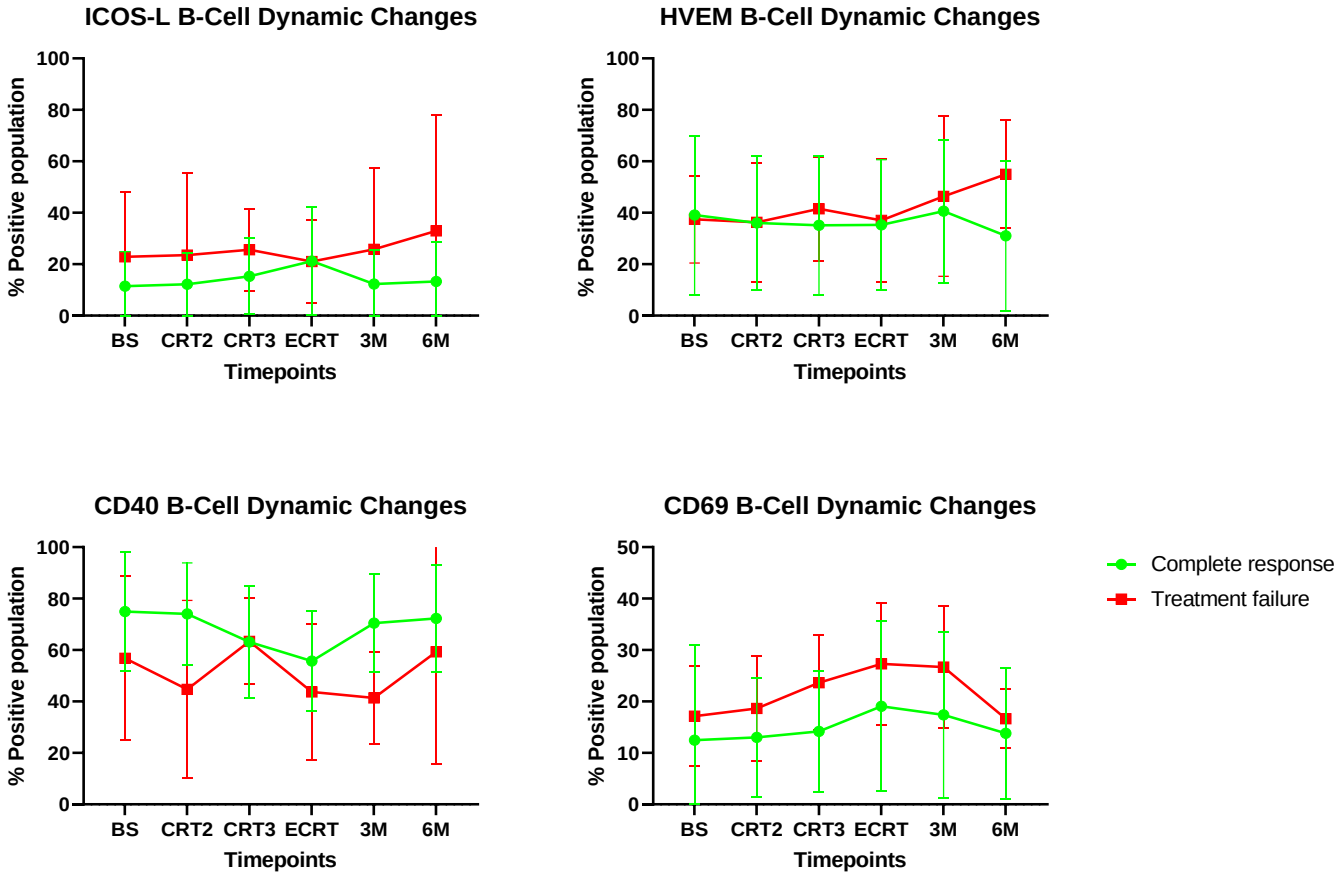


Figure 3.62 Dynamic changes in expression of ICOS-L, HVEM, CD40 and CD69 Expression on B-cells. Data is expressed as % positive of B-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

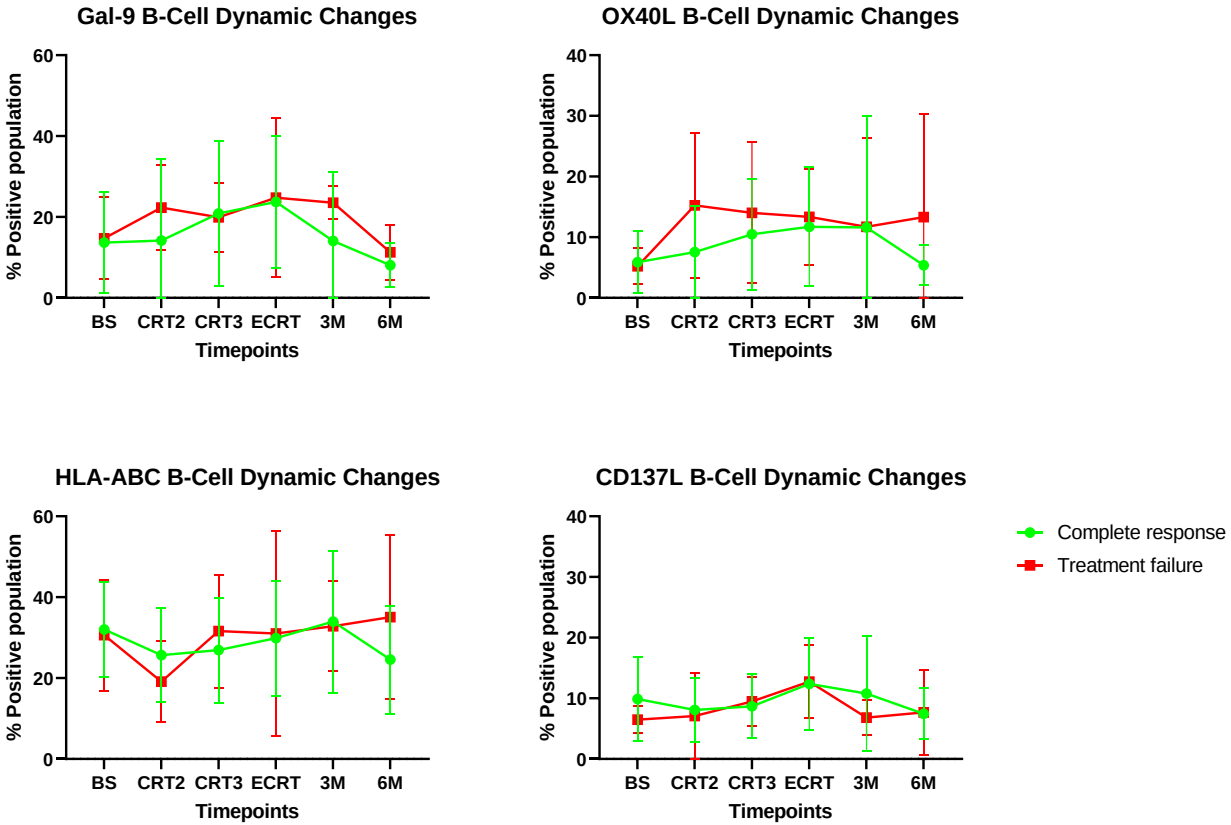


Figure 3.63 Dynamic changes in expression of Gal-9, OX40L, HLA-ABC and CD137L Expression on B-cells. Data is expressed as % positive of B-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

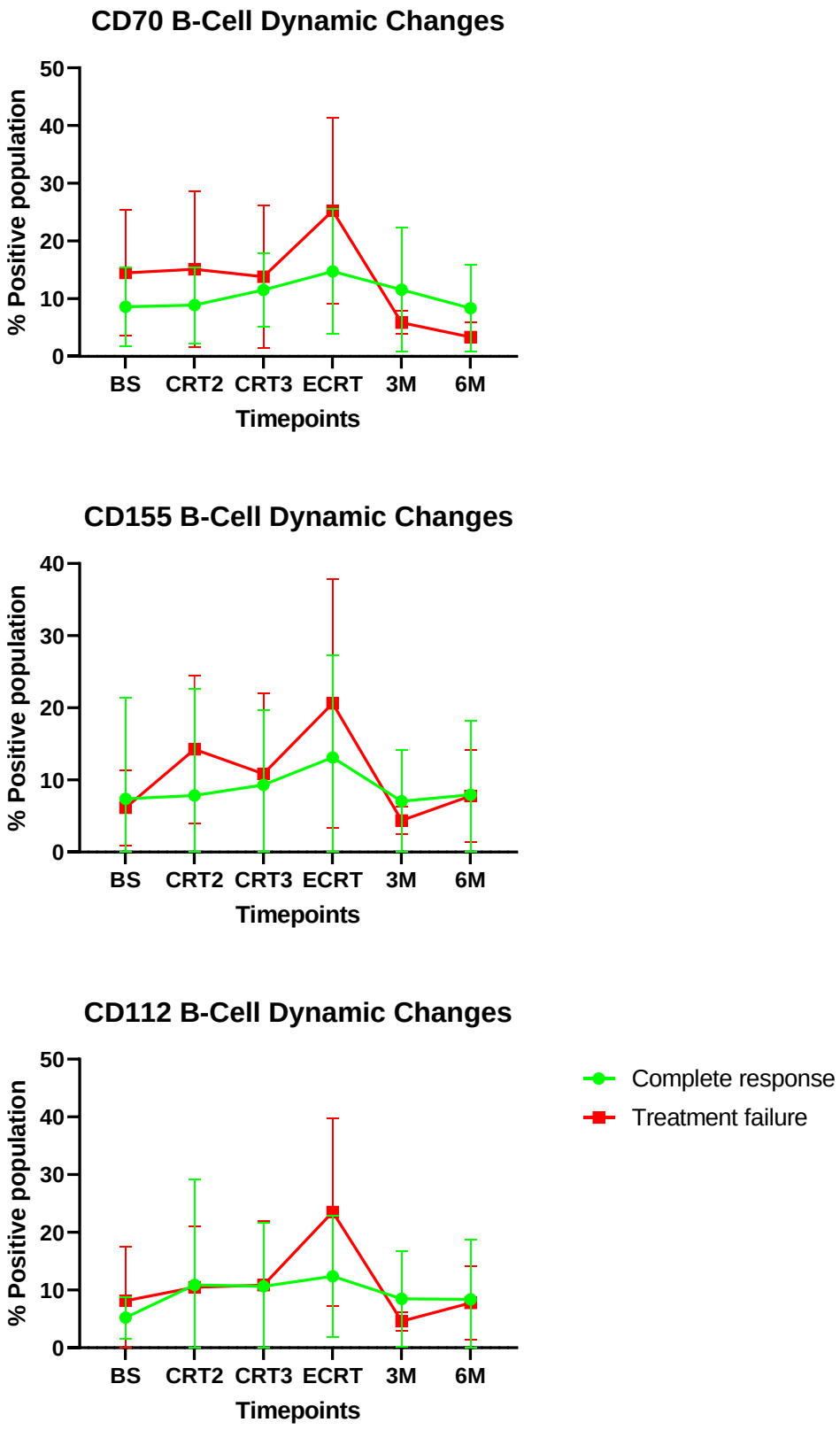


Figure 3.64 Dynamic changes in expression of CD70, CD155 and CD112 Expression on B-cells. Data is expressed as % positive of B-cell population +/-SD. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent

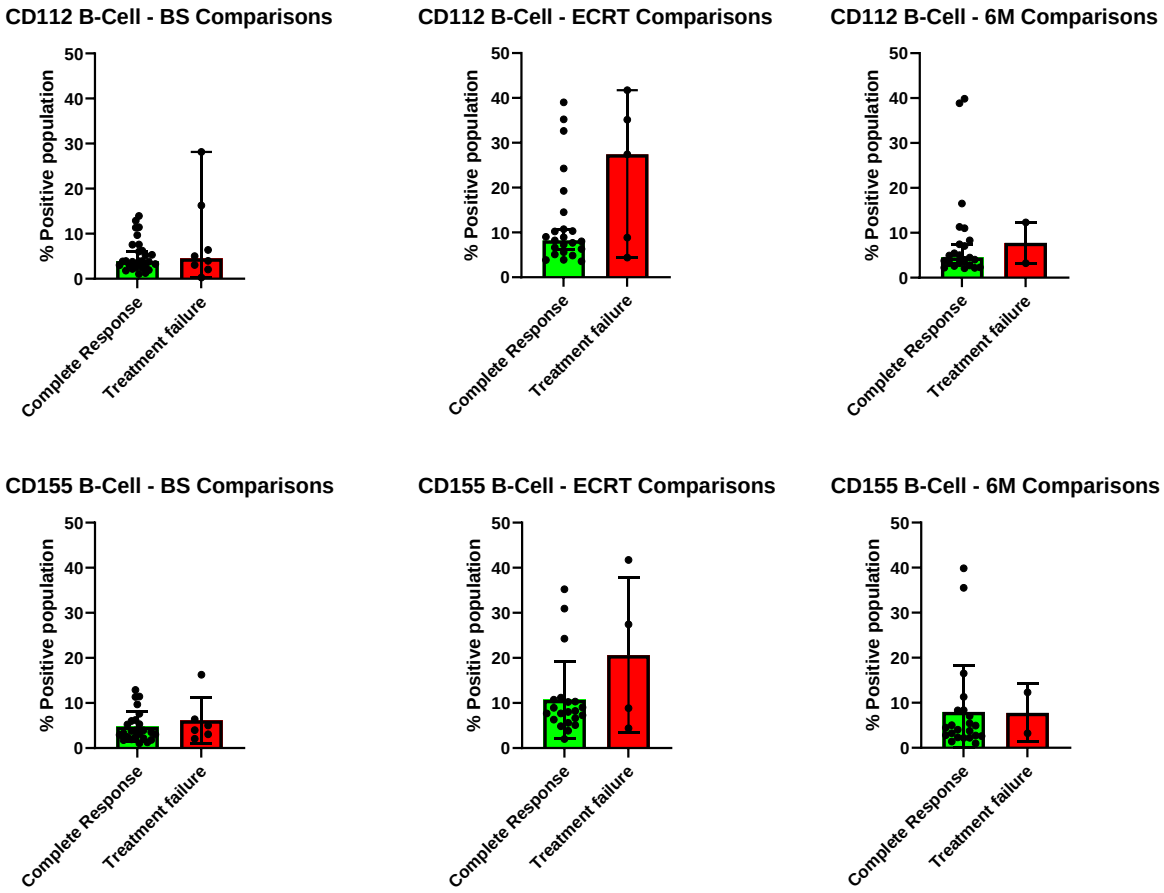


Figure 3.65 Comparison between complete response and treatment failure groups for CD112 and CD155 on B-cells. Data is expressed as % positive of B-cells population with 95% Confidence Intervals. Data is presented for N=25-30 (CR); N=3-8 (TF), sample availability dependent.

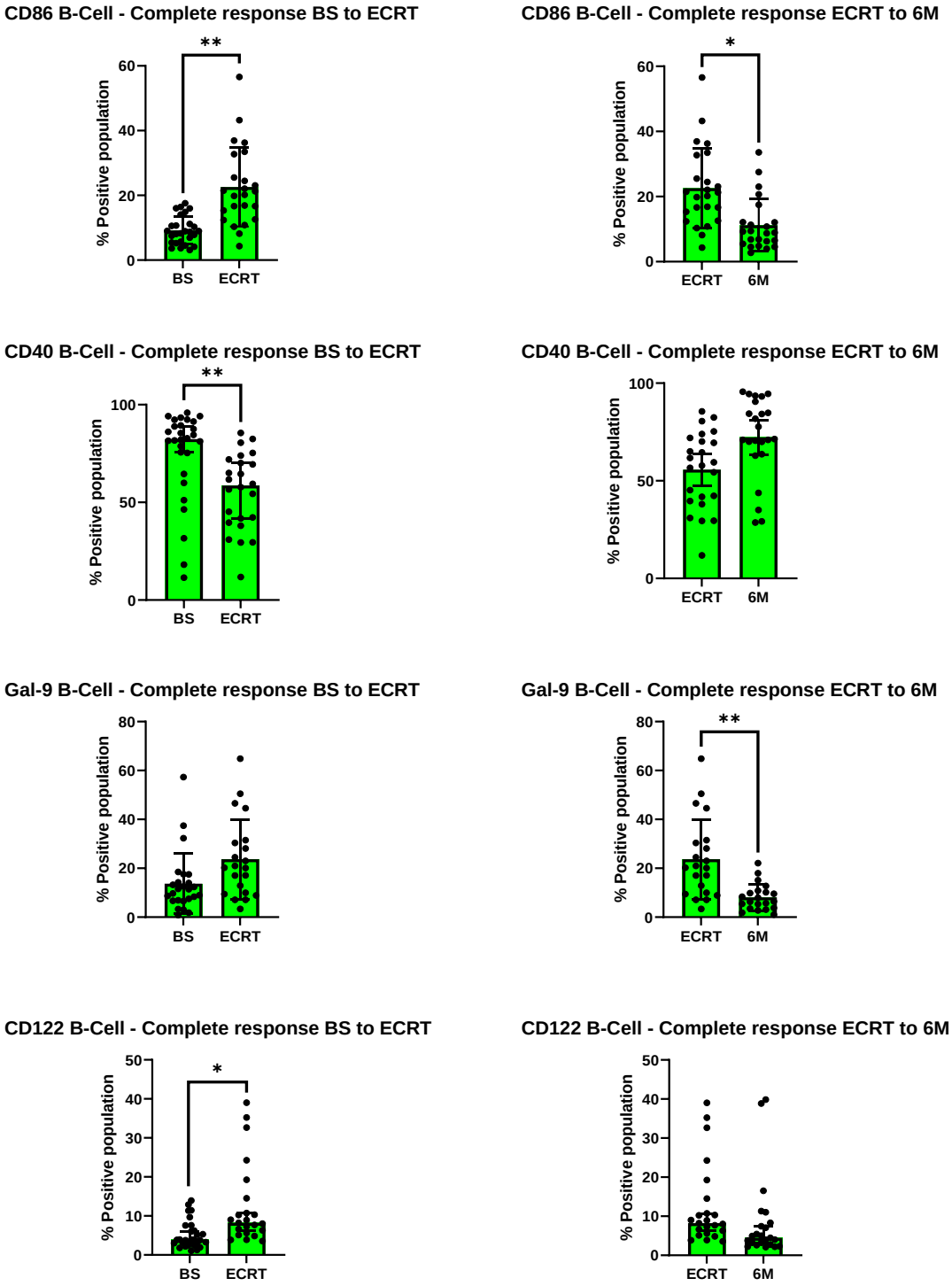


Figure 3.66 Changes CD86, CD40, Gal-9 and CD112 Expression on B-cells from baseline to end of treatment and end of treatment to 6 months for complete response. Data is expressed as % positive of B-cells population +/-95%CI for non-parametric. Data is presented for N=30 (BS); N=25(ECRT and 6M). ✱P ≤ 0.05, ✱✱P ≤ 0.01

3.19 Discussion

This chapter used flow cytometry with a large immunophenotyping panel on serial PBMC blood samples from the GRECIAN study to investigate the cellular markers and dynamics of immune resistance to CRT for locally advanced ASCC.

Checkpoint inhibition targeting PD-1/PD-L1 has been most successful in RT-IO trials in other tumour sites. CD8+ T cells are considered the most important cell type for checkpoint inhibitors due to their direct cytotoxic effects on tumour cells, elevated PD-1 expression in their exhausted state, and the link between CD8+ TILs and IO response. However, many other cell types are essential. For example, CD4+ Helper T-cells activate and maintain CD8+ T cells, and DCs engage in priming and activating T cells. NK cells can be directly cytotoxic themselves, express PD-1 and produce cytokines that enhance T-cell response. Tregs are essential in controlling immune responses. Given the complex interactions between different cell types in eliciting and maintaining an immune response, a large immunophenotyping panel identifying multiple cell types was used. Furthermore, given the ever-expanding repertoire of available drugs that target immune proteins other than PD-1/PD-L1 and CTLA-4, multiple proteins were investigated within each cell type. To our knowledge, this is the first serial immunophenotyping of ASCC patients receiving standard-of-care CRT.

A sizeable statistical analysis plan resulted in numerous statistically significant differences despite adjustments. Differences were observed between patients with a complete response to CRT and those who experienced treatment failure by 6 months, as well as dynamic changes within these patient groups, often showing increased expression during CRT, followed by a return towards baseline 6 months after treatment finished.

3.19.1 Immune cell populations

Within immune cell population analysis, the association between high peripheral Treg population and treatment failure was the most exciting result. Patients with treatment failure had significantly higher peripheral Tregs at baseline, with a trend for higher Tregs at ECRT and 6M. In both patient groups, there was a trend for peripheral Tregs to increase during treatment and then decrease 6 months after treatment finished.

Similar results for the association between treatment outcome and baseline peripheral Tregs were published by Fokas et al. in a similar cohort of patients receiving CRT for ASCC[185]. As part of a more extensive analysis plan investigating IHC, RNAseq, and peripheral cytokine analysis, they performed flow cytometry on 47 patients, showing an association between higher peripheral Tregs at baseline and LRF/metastatic disease. In the Fokas study, Tregs were classified as CD4+CD25+FoxP3+ and they classified “High Tregs” as representing more than 5.2 % of CD3+ T-cells. This gating strategy is less

specific to Tregs than the one used in this study, but when this gating strategy was used on the GRECIAN dataset, similar results were observed. Data on Treg dynamics was not reported in the Fokas study and has not been reported for ASCC elsewhere.

Tregs are recognised as the most radioresistant subset of lymphocytes, a finding replicated in GRECIAN showing a shift towards FOXP3+ as a proportion of viable cells[186]. They are the only subset of lymphocytes that showed a stable or slight increase during treatment. Similar dynamics have been reported in other cancers. In lung cancer, 24 patients with locally advanced NSCLC receiving CRT had blood taken before treatment, every week during CRT and 1 month post-CRT[187]. There was no significant change in Tregs as a proportion of CD4+ T-cells in response to CRT, with a drop in total T-cells and NK cells during treatment, which recovered post CRT. A separate study in 10 patients receiving SBRT for liver cancer found no change in CD4+CD127-CD25+ cells as a proportion of CD4+ T-cells but a decrease in CD3+, CD4+ and CD8+ T-cells and NK cells in response to treatment[188].

Outside of response to RT, the reported prognostic value of Tregs in peripheral blood at diagnosis is mixed. Higher peripheral Tregs have been shown to correlate with poor outcomes in metastatic melanoma but were associated with better response in 62 patients treated with ICIs for various cancers[189,190].

Most of the literature on the prognostic value of Tregs in cancer relates to FOXP3+ TILs, where results are also conflicting. A large meta-analysis from 2015 found that overall, FOXP3+ TILs had a negative effect on survival but varied greatly between tumour sites[191]. FOXP3+ TILs had a negative association with survival in CC, whilst in HNSCC and colorectal cancer FOXP3+ TILs had positive association with survival. Even within tumour sites, results varied greatly; in breast cancer, four studies found a negative prognostic value for high FOXP3+ TILs, six found a neutral effect, and three found a positive effect on survival. No studies of ASCC were included in this meta-analysis.

One explanation for the heterogeneity in results within and between tumour sites is inconsistent Treg classification. A 2018 systematic review identified 45 papers examining Tregs in HNSCC, 55.6 % of which correlated Tregs to poor clinical outcomes, 20 % to good outcomes, and 24.4 % did not reach a conclusion[192]. In the nine studies that suggested a positive outcome, only 5 used FOXP3 to identify the Tregs. FOXP3 is also highly expressed in other activated T-cell subsets, including effector T cells[193]. If a single IHC stain for FOXP3 is used, it may be a marker of activation rather than regulation. FOXP3 is also expressed by cancer cells, where it has been reported to be associated with poor outcomes and has different biological functions compared to FOXP3 in Tregs[194]. The authors suggest dual IHC staining for CD4 and FOXP3 could better identify true Treg TILs.

In ASCC, FOXP3+ TILS have been associated with superior DFS[33]. The association between poor outcomes with high peripheral Tregs and good outcomes with high

FOXP3+ TILs is complex and requires further exploration. More specific classification of peripheral and tumour infiltrating Tregs may help clarify this relationship.

Assuming that high baseline peripheral Tregs are a poor prognostic marker for response to CRT in ASCC, what are some approaches to reduce them before CRT? Low dose cyclophosphamide could be a low-cost option. In one study, patients with metastatic breast cancer received 3 months of daily 50mg of cyclophosphamide, with a substantial drop in peripheral Tregs after 14 days, with minimal toxicity[195]. In another, patients with metastatic colorectal cancer who received 50mg cyclophosphamide days 1-7 and 15-21 over a 22-day course with metastatic colorectal cancer showed induced Treg depletion by day 15, with an associated delay in tumour progression[196]. This led to the ongoing BICCC (Brief Intervention with Cyclophosphamide in patients with Colorectal Cancer) trial, which uses 4 weeks of low dose cyclophosphamide after chemotherapy/surgery[197]. In ASCC, a short course of cyclophosphamide before CRT could be given between the RT planning scan and initiation of CRT. This would not delay the current patient pathway and allow translational blood to be taken to assess peripheral Treg response to cyclophosphamide.

While the total CD4+ T-cell population offers valuable insights, its functional and phenotypic diversity necessitates cautious interpretation of results. Among CD4+ T cells, CD4+ Helper T cells comprise the majority, representing approximately 65–80% of the total population[198]. However, Tregs constitute about 5–10% of the total CD4+ T-cell population and have different and often opposing function[199].

It was unfortunate that the DC immunophenotyping did not work, and this does not mean that they are not important for the immune response of ASCC. However, it does suggest that peripheral measurement of DCs in the future is unlikely to be a successful prognostic or predictive biomarker.

3.19.2 Immune cell markers

As expected, the majority of markers investigated showed the FOXP3+CD4+CD25^{high}CD127^{low} population in GRECIAN exhibited an inhibitory phenotype compared to the FOXP3-CD4+ population. The one exception was the expression of PD-L1, which was higher in the FOXP3-CD4+ population. Whilst unexpected, this could be because the Treg population suppresses other T-cells through multiple mechanisms including direct contact, whilst the FOXP3-CD4+ population is immune exhausted itself. Within this FOXP3+CD4+CD25^{high}CD127^{low} inhibitory phenotype, Tregs in patients with treatment failure showed higher CTLA-4 expression at baseline than in patients with complete response, with expression increasing throughout treatment. There was also a trend for higher TIGIT expression on Tregs in treatment failure, but this did not reach significance. Therefore, not only was there a higher proportion of Tregs in patients with treatment failure, but these Tregs themselves were more inhibitory. In the CD4+ T-cells, CD8+ T-cells and NK cells, TIGIT

and CTLA-4 were the markers most associated with treatment failure, generally at baseline.

CTLA-4 plays an important role in the suppressive function of Tregs, competing with CD28 for binding with CD80/86. Many trials have tried combining RT with CTLA-4, usually using first-generation drugs such as ipilimumab, with limited success[200,201]. However, newer CTLA-4 drugs such as botensilimab bind more strongly to activating Fc-γ receptors, depleting intratumoral Tregs through antibody-dependent cellular cytotoxicity, and could be therapeutic options for future ASCC RT-IO trials[202]. In GRECIAN, there was no difference between treatment failure and complete response for CD28 expression on Tregs, or CD80/86 on monocytes.

TIGIT expression was particularly high across all cell types in GRECIAN. TIGIT blockade has been shown to enhance anti-tumour T-cell response to RT via CD8+ T-cells in an oesophageal cancer mouse model [203]. In a syngeneic breast cancer model that was considered immunological cold, anti-TIGIT+anti-PD-1+RT resulted in local and systemic immune responses, delaying tumour growth and metastatic spread[204]. There are numerous new anti-TIGIT antibodies currently in clinical trials, including tiragolumab which has positive Phase III data in advanced NSCLC[199]. There is currently no published data on TIGIT-RT combination studies in humans, but there are currently two ASCC trials investigating anti-TIGIT drugs. TIRANUS is a Phase II single-arm trial investigating concurrent and adjuvant atezolizumab and tiragolumab with SoC CRT in locally advanced ASCC, recruitment is ongoing[205]. Another trial ran from MD Anderson is using an anti-PD-L1/TIGIT combination for patients with HPV-associated minimal residual disease (HNSCC, ASCC, cervical or penile) after curative-intent treatment[206].

Data from monocyte marker expression supports TIGIT as a possible immune resistance mechanism. CD112 and CD155 are two TIGIT ligands expressed on tumour cells and APC. CD112 expression on monocytes at baseline was higher in patients with treatment failure compared to complete response, with a trend for higher expression at ECRT and 6M. There was a small trend for higher CD155 expression on monocytes at baseline in treatment failure. CD155 expression was already very high in the complete response, and similar statistical results were seen in CD155 when using MFI for monocytes instead of % expression. TIGIT is a co-inhibitory molecule that competes with the co-stimulatory CD226 (DNAM-1) for binding with CD155 and CD122 in a similar mechanism to the CD28/CD80/CTLA-4 system. CD155 has been shown to be associated with radioresistance in squamous oesophageal cell lines[207]. There are no data on CD155 or CD112 in ASCC, and using IHC on these patient samples would be a good first step in this area, as well as looking at expression in the 5 ASCC cell lines. In future peripheral flow cytometry studies, it would be helpful to look for DNAM-1 expression.

Although most of the significant results were from the targeted analysis, there were some unexpected results in the exploratory analysis. Higher TIM-3 expression on CD8+ cells was associated with treatment failure. Similar results were found in locally advanced nasopharyngeal cancer, where co-expression of TIM-3 and TIGIT on peripheral CD8+ T-cells was associated with poor prognosis[208]. TIM-3 has been shown to promote Tregs to inhibit CD8+ T-cell in HPV-associated CC[209]. Further assessment of TIM-3 in future translational studies is warranted.

3.19.3 Timing of immunotherapy

When comparing the dynamics of these immune markers to the hypothetical examples given at the start of this chapter, a neoadjuvant approach seems the most reasonable to overcome the three main resistance markers identified – peripheral Tregs, TIGIT and CTLA-4. Although they all increase to varying degrees in response to CRT and then decrease afterwards, there is little difference in this response between responders and non-responders. This applied pattern applied other co-stimulatory and co-inhibitory markers.

Given the differences observed between patient groups for CTLA-4 and TIGIT, the most surprising result was PD-1 and PD-L1 expression. Although there was a clear response in both markers across immune cell subtypes to CRT, there was no difference between the groups. This could be an explanation for why some of the PD-1/PD-L1 RT-IO trials in HNSCC and CC have not had positive results

3.19.4 Other literature

The results presented here are comparable to the small translational immunology literature in ASCC. The Fokas study has the closest patient population to GRECIAN and found PD-1 expression on CD4+ cells increased during treatment in all patients, although the decrease after treatment observed in GRECIAN did not occur. TIGIT and CLTA-4 were not investigated. A 2024 ASCO GI abstract investigated circulating immune biomarkers from NCI9673, a trial that investigated nivolumab with or without ipilimumab for metastatic ASCC[210]. They found mean expression of TIGIT was highest relative to other immune checkpoint biomarkers (PD-1, CTLA-4, TIM-3, LAG-3, TIGIT, OX40 and ICOS), a result replicated in GRECIAN. This study was the reason for including TIGIT in the targeted markers for statistical analysis in GRECIAN.

3.19.5 Strengths and weaknesses

The depth of analysis for multiple different immune markers and cell types and the relatively tight classification of Tregs compared to previously published work is a strength of this analysis. Assessment of dynamic changes in peripheral blood over time give more detailed information on optimal timing than many prior publications. Many prior studies do not split their results by treatment outcome, which is essential for designed future precision medicine trials. Although serial tumour sampling studies for

RT-IO combinations are feasible for some tumour sites, as used in the PRIME-RT study in rectal cancer, this is not practical for many tumours[211]. A repeat on-treatment biopsy was initially considered for GRECIAN, but the Leeds PPI group felt it would be far too painful and would require general anaesthetic to go ahead, making it ethically and financially unviable. Repeated on-treatment biopsy is part of the CORINTH ASCC trial, but not a single patient at any recruiting centre has had it due to local PI concerns (personal communication). Alternatively, repeated imaging could be used to assess response in the tumour during CRT. There are multiple radiomics approaches to assessing TILs, PD-1 expression and CTLA-4 expression published that could be used[212,213].

Lack of tissue and associated immune cell-tumour cell interaction is a weakness of the data present. Although not presented as part of the thesis, there is planned and ongoing work on the corresponding tissue using multiplex-IHC and RNAseq to assess how these peripheral blood findings correlate with the TME.

Although not presented, all patients in this study had varying degrees of lymphopenia during treatment that impacted total PBMC cell counts from each blood sample. This makes interpreting the results more challenging. The dynamic changes in immune cell populations were presented as a relative proportion of viable cells, but the absolute number of these cells present per ml of blood is not considered. How important is it that there is a relative increase in Tregs as a proportion of the lymphocyte population when the patient has a substantially lower total number of lymphocytes, and thus a substantially lower total number of Tregs? This is a tricky question to answer. This lymphopenia may explain why previous concurrent RT-IO trials have not worked, as there are no immune cells with which the IO can interact.

The statistical methods used here could be described as unnecessarily complex. Given the relatively low numbers in the treatment failure group and the method used by Benjamin-Hochberg for p-value adjustments, many trends that appear to show large magnitudes of difference between complete response and treatment failure have comparatively higher p-values. This could suggest the presence of Type II errors. However, the consistency with which TIGIT and CTLA-4 were found to be significant across multiple cell types, their relative importance in Treg suppressive function, the substantial difference seen in Tregs between groups and the signal of a difference in CD112/CD155 on monocytes support these three immune markers as avenues to explore in future trials. Targeting Tregs, CTLA-4, or TIGIT in the neoadjuvant setting is a reasonable option for a future RT-IO ASCC study.

There are many areas steps in flow cytometry analysis that have the potential to alter the result. For example, a decision on whether to include % expression of a marker at a particular timepoint that is much higher or lower than its peers is difficult to make. Is it a true reflection of expression or an issue with sample processing? If the sample were

of good quality, other markers in that sample fit the FMOs, the same marker looks good on other timepoints and the split looks clear on that plot, that would favour inclusion. To reduce the risk of a type II error, I have erred on including these samples. Non-parametric tests have been used to account for a few of these results that have been included, which reduces the power of the test.

This heterogeneity in flow cytometry results is more likely when testing in multiple patients compared to animal work using genetic clones of the same mouse. This inherent heterogeneity contributed to the wide confidence intervals seen, weakening the statistical tests used. However, the confidence intervals are comparable to similar published studies, such as Fokas's study in ASCC.

There is a risk of “future leakage” from this type of analysis, when information is used to assign a likely outcome that is only known after the event has occurred is available. For example, if differences in peripheral Tregs only occurred at 6 months after treatment finished, there would be no way of knowing which patient this would occur to at the start of treatment. Fortunately, given the results this risk of biased stratification is minimised, as all of the immune resistance markers identified were detectable at the start of treatment.

Given the number of patients in this study, a binary analysis comparing treatment failure versus complete response was chosen. However, there are other ways this data could have been classified. At its most granular, the patient response could have been classified as ordinal data – starting with complete response at 3 months, then complete response at 6 months, persistent disease at 6 months, progressive local disease at 6 months, progressive disease at 3 months, metastatic disease at 6 months and metastatic disease at 3 months. Within the treatment failure group, there were generally higher Tregs and higher expression of co-inhibitory markers in those that had early metastatic relapse compared to those with persistent disease, but the numbers do not allow for any valid conclusions to be drawn.

3.19.6 Future work

Ongoing and planned RNAseq and multiplex-IHC will be used to confirm the importance of Tregs in the tumour itself. In particular, CD155 and CD112 expression in tumour tissue could help confirm the TIGIT/CD155/CD112 axis as a target for future trials. Assessment of CD155/CD112 expression on the recently acquired PMAC ASCC cell lines and its correlation with the radioresistance of each line could provide further evidence[97].

A more targeted immunophenotyping panel that gives a more precisely and examines DNAM-1 expression would further detail the immune resistance mechanisms identified. Multiple Treg populations with different functions could be characterised using CD39, CD45, and Helios[214,215]. To characterise the non-concordant findings

discussed earlier, multiplex-IHC could be performed on PBMCs and FFPE tissue. If the results from this tissue work are promising, they could be explored using the FFPE diagnostic tissue collected as part of ACT5.

3.20 Conclusion

The overall aim of this chapter was *"To identify pre-CRT and CRT-induced cellular markers of ASCC immune resistance and to correlate these with 6-month treatment failure (circa 25-30 %) and complete response (circa 70-75 %)".* The analysis presented aimed to identify differences in complete response and treatment failure for ASCC patients receiving CRT, whilst also evaluating dynamic changes in these immune markers over time.

8 (20 %) of the 40 patients recruited to GRECIAN had treatment failure at 6 months. Differences in Tregs, CTLA-4 expression, and TIGIT expression were observed prior to the initiation of CRT. These results suggest that targeting these with a neoadjuvant approach through low-dose metronomic cyclophosphamide, botensilimab, or tiragolumab could be utilised in future RT-IO trials for locally advanced ASCC.

Chapter 4 Soluble markers of immune resistance to chemoradiotherapy for locally advanced anal cancer

4.1 Introduction

This chapter investigated the soluble immune resistance markers from standard of care CRT for locally advanced ASCC. The investigated soluble markers were assayed in plasma.

ELISA is a well-established method for measuring cytokines and chemokines in plasma or serum. Multiplexing allows for multiple ELISA tests in one sample. It is a type of bead-based assay that uses different proportions of two fluorophores coated on each bead to correspond to a specific capture antibody. The cytokine/chemokine of interest binds to the capture antibody, and biotinylated detection antibodies specific to each analyte of interest are added, followed by streptavidin-phycoerythrin (Strep-PE). Samples are then acquired using lasers that can identify each bead according to the specific ratio of the two fluorophores and measure their quantity, using the intensity of the PE signal. This technique was used to measure 48 markers of interest in one well in plasma from the GRECIAN cohort. Given the pre-sample processing required for TGF-β, the 3 isoforms of TGF-β were measured separately but using the same Multiplex technology.

While many cytokines are thought to play a role in RT and IO responses, none have been approved for combination treatment with RT. While IL-2 and IFN-γ have previously been approved as monotherapies for various cancers, they are no longer used due to their significant side effects[125]. Galunisertib, a TGF-β type I receptor kinase inhibitor, showed improved response rates in rectal cancer in combination with CRT[127]. There are ongoing trials combining RT with TGF-β and other cytokines treatments to improve response rates[216].

IFN-γ and TNF-α are specific cytokines thought to play important roles in the immune response to radiotherapy (RT). These cytokines belong to families of signalling molecules that often function in a coordinated manner - for example IFNs comprise IFN-γ, -α, and β. IFNs can be further classified into type 1 which all bind at a common cell-surface receptor, and type 2, which bind at distinct receptors. Furthermore, different cytokines can have overlapping function even if they are not on the same pathway -– for example TNF-α and TRAIL act on separate receptors but can influence each other's receptor expression. TNF-α and TRAIL can both trigger cell apoptosis but do so through different but overlapping pathways[217–219]. This shows that the effects of specific cytokines are rarely isolated. The dynamic responses of cytokines to CRT in ASCC are unknown. Given this, a combination of cytokines known to be relevant in radiotherapy and IO, as well as cytokines in their associated families and ligands were measured.

4.2 Patient samples available for analysis

Given the simplicity with which plasma is collected compared to PBMCs, all 210 samples detailed in Table 2.1 had plasma available for analysis.

4.3 Statistical methods – considerations and plan

As many of the cytokines investigated belong to the same family or are part of the same pathway, they have an inherent interdependence, which makes interpretation more difficult. Given the large number of cytokines measured, if the same adjustment principle as Chapter 3 were applied here, it could result in over-correction and increase the risk of a type II error. Given that most of these cytokines have not been measured in this way in ASCC before, all data are exploratory, and no adjustment was performed. Significance was set at $\alpha<0.05$.

The data produced by Multiplex assays is expressed as pg/ml (dependent quantitative variable) and how it changes according to response outcome (independent categorical variable) and timepoint (independent categorical variable). The following statistical comparisons were performed given the chapter's aims, the type of data available and the statistical considerations described.

1) Baseline comparisons between groups (between complete response versus treatment failure)
2) ECRT comparisons between groups
3) 6M comparisons between groups.
4) Comparison within groups, comparing BS versus ECRT (for example, baseline complete response versus ECRT complete response)
5) Comparison within groups, comparing ECRT versus 6M.

Data distribution was measured at baseline for each cytokine. The Student's T-test or Mann-Whitney U test was used for comparisons depending on the data distribution. Sensitivity analysis was used to aid this decision by comparing p-values from comparative tests, data transformation, and removal of outliers.

Dynamics changes for all cytokines are presented. Individual comparisons for all significant results are shown, as well as any results deemed to have biological relevance.

4.4 Results

Unfortunately, many cytokines investigated were either undetectable or present in very low levels in the first 48-plex human cytokine plate. This was performed using the manufacturer’s recommended dilution of 2:1. The assay was, therefore, repeated without any dilution of samples. However, many cytokines remained undetectable.

Given these results and the cost of running each plate, it was decided not to repeat this experiment with the remaining samples. Results are presented for 14 patients – 11 with a complete response and 3 with treatment failure. Undetectable results have been recorded as 0 pg/ml. All measurements for IL-2, IL-5, IL-15, and VEGF were undetectable and results are not presented. The TGF-β plate was run three times. Given the cost and lack of early signal of difference between complete response and treatment failure, it was decided to not to run the rest of the samples. Results are presented for 19 patients –12 with complete response and 7 with treatment failure.

Markers have been broadly categorised by their function, recognising there is substantial overlaps for many of the cytokines and chemokines measured. Figure 4.1 shows dynamic changes in anti-inflammatory cytokines. Figure 4.2 andFigure 4.3 show dynamic changes in pro-inflammatory cytokines. Figure 4.4 andFigure 4.5 show dynamic changes in chemokines. Figure 4.6 andFigure 4.7 show dynamic changes in growth and haemopoietic factors. Figure 4.8 and Figure 4.9 show dynamic changes in the remaining interleukins. Figure 4.10 shows dynamic changes for TGF-β isoforms 1, 2 and 3. As there was only one sample at 6M for treatment failure some between group comparisons were not possible. Although changes over time were detectable in many markers, low numbers and substantial variability meant that no between-group comparisons had statistically significant results.

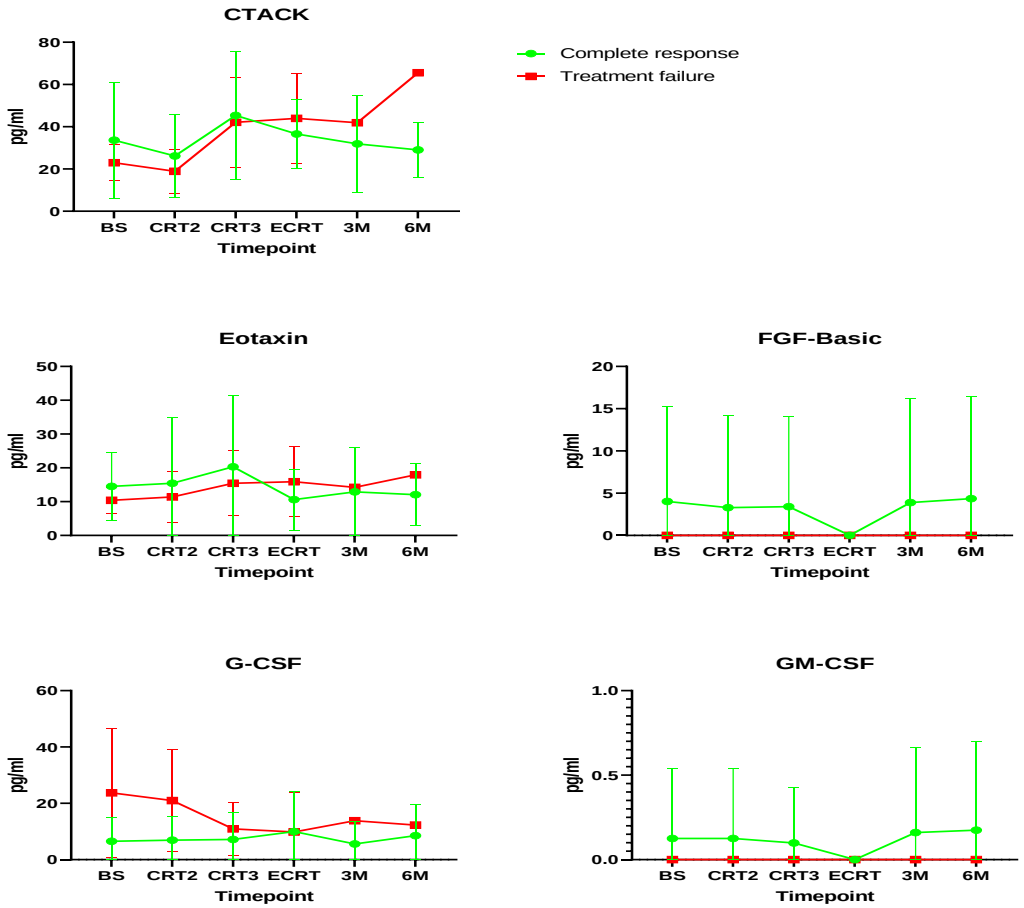


Figure 4.1 Dynamic changes in Anti-inflammatory cytokines IL-1ra, IL-4 and IL-10 for complete response and treatment failure. Data is expressed as pg/ml +/-SD. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

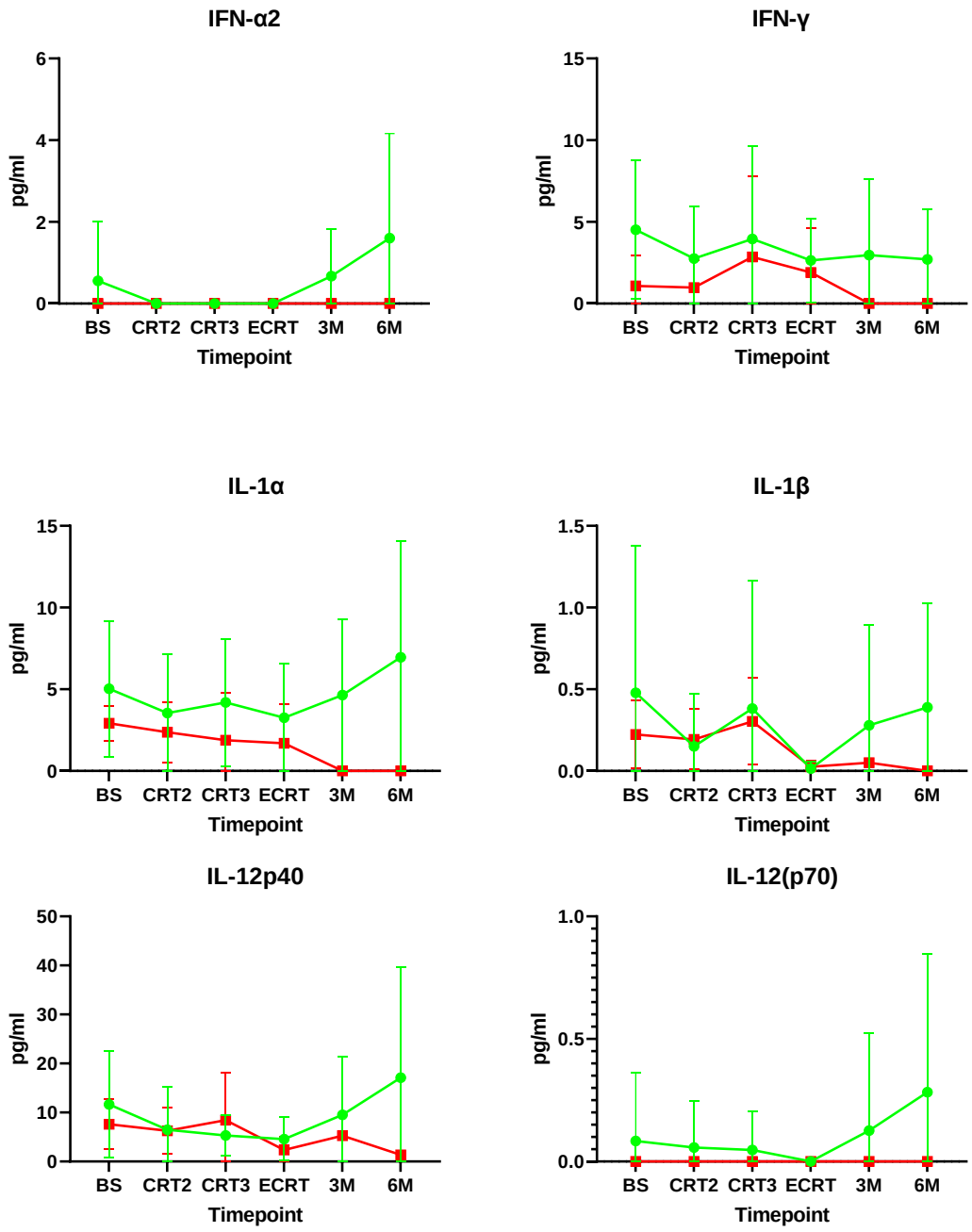


Figure 4.2 Dynamic changes in pro-inflammatory cytokines IFN-α2, IFN-γ, IL-1α, IL-1β, IL-12p40 and IL-12(p70) for complete response and treatment failure. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

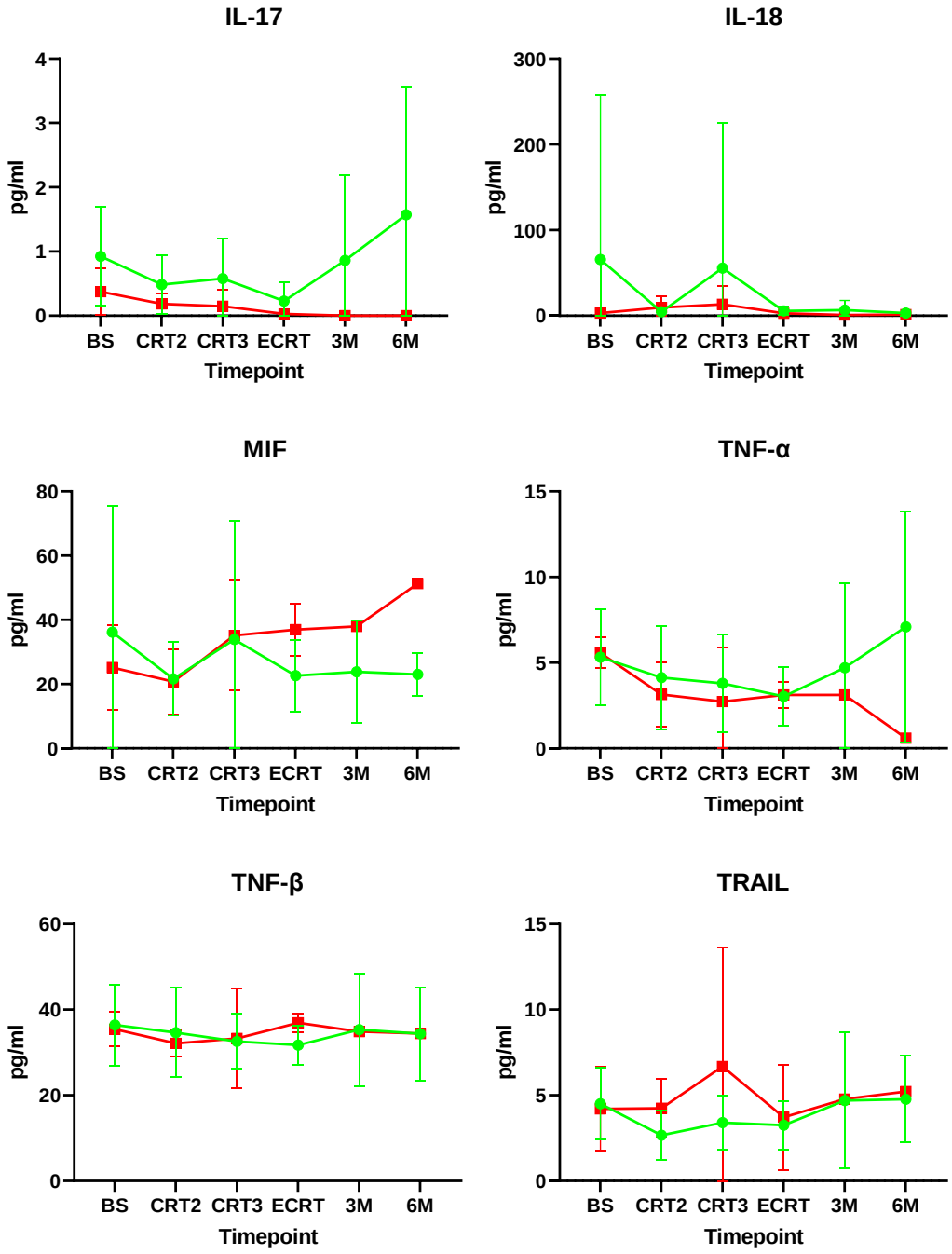


Figure 4.3 Dynamic changes in pro-inflammatory cytokines IL-17, IL-18 MIF, TNF-α, TNF-β and TRAIL for complete response and treatment failure. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

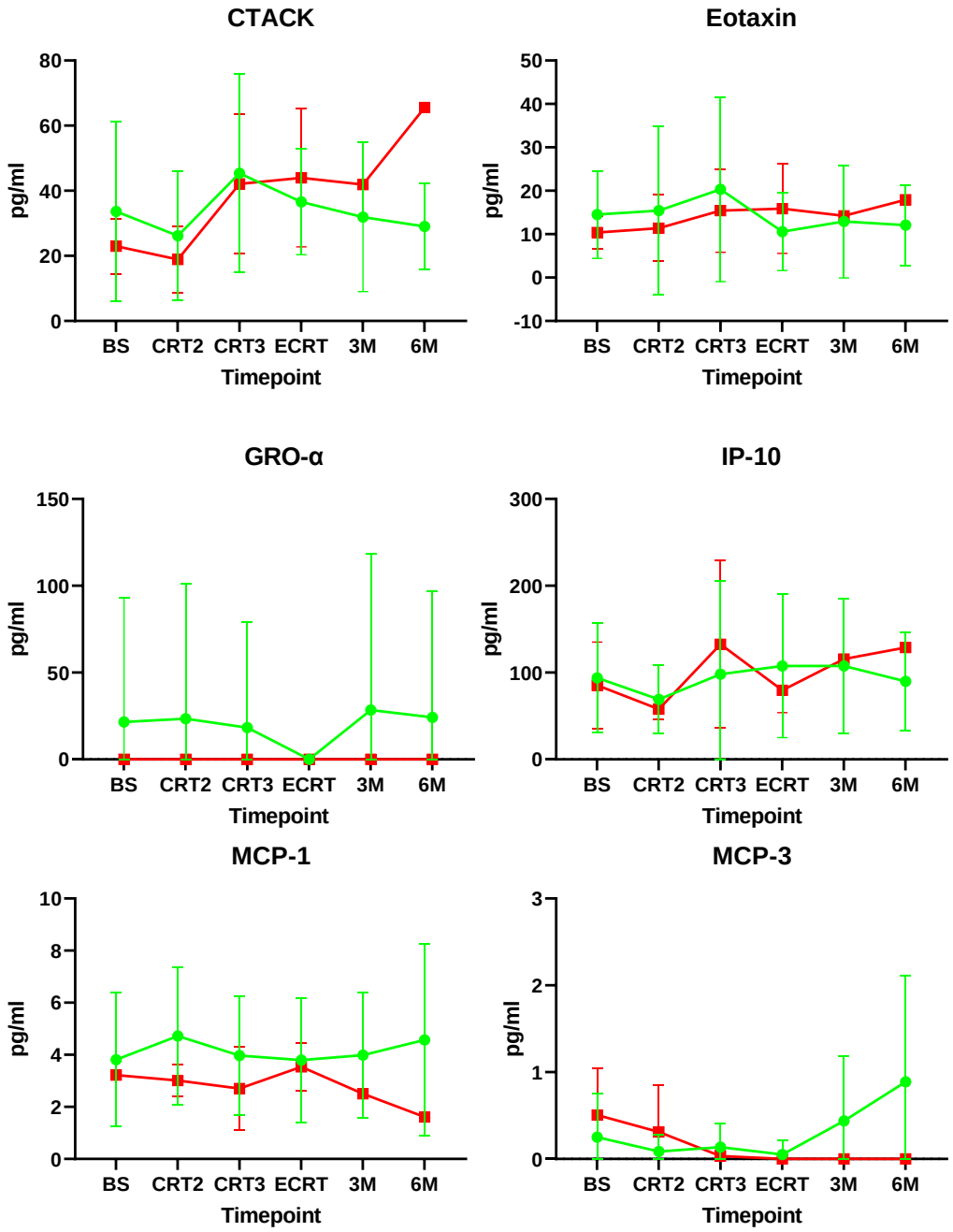


Figure 4.4 Dynamic changes in chemokines CTACK, Eotaxin, GRO-α, IP-10, MCP-1, and MCP-3 for complete response and treatment failure. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

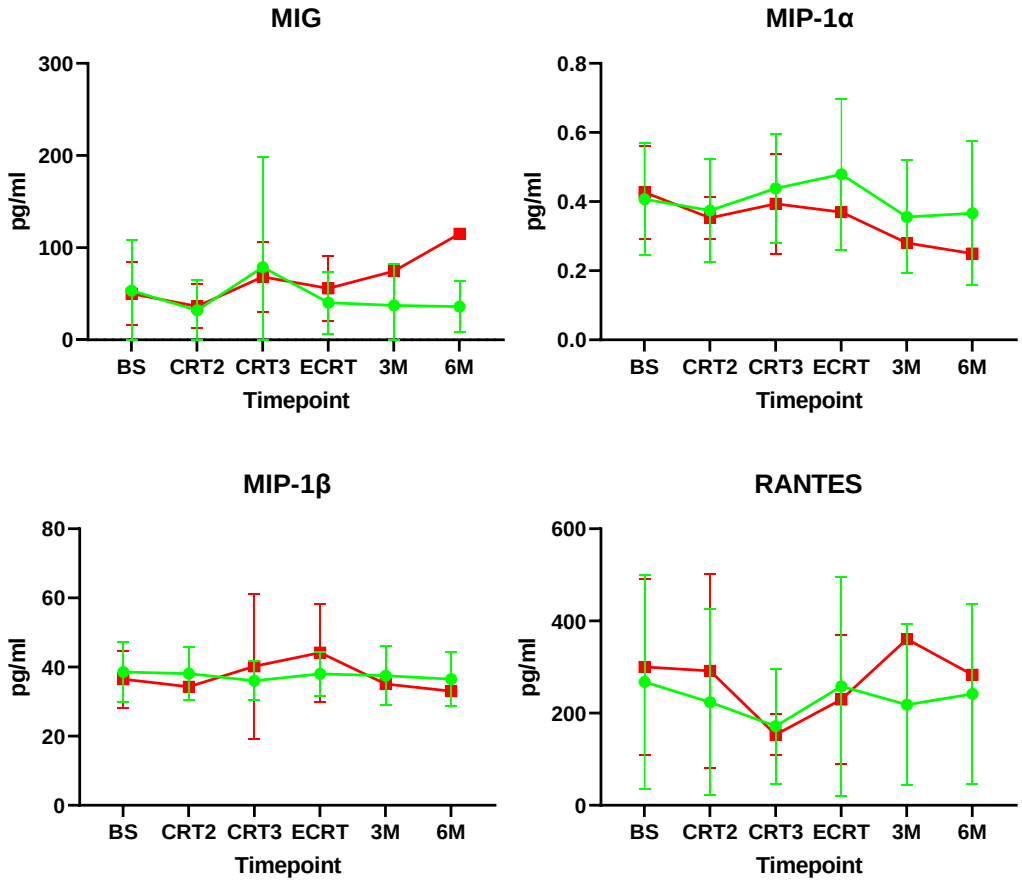


Figure 4.5 Dynamic changes in chemokines MIG, MIP-1α, MIP-1β, and RANTES for complete response and treatment failure. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

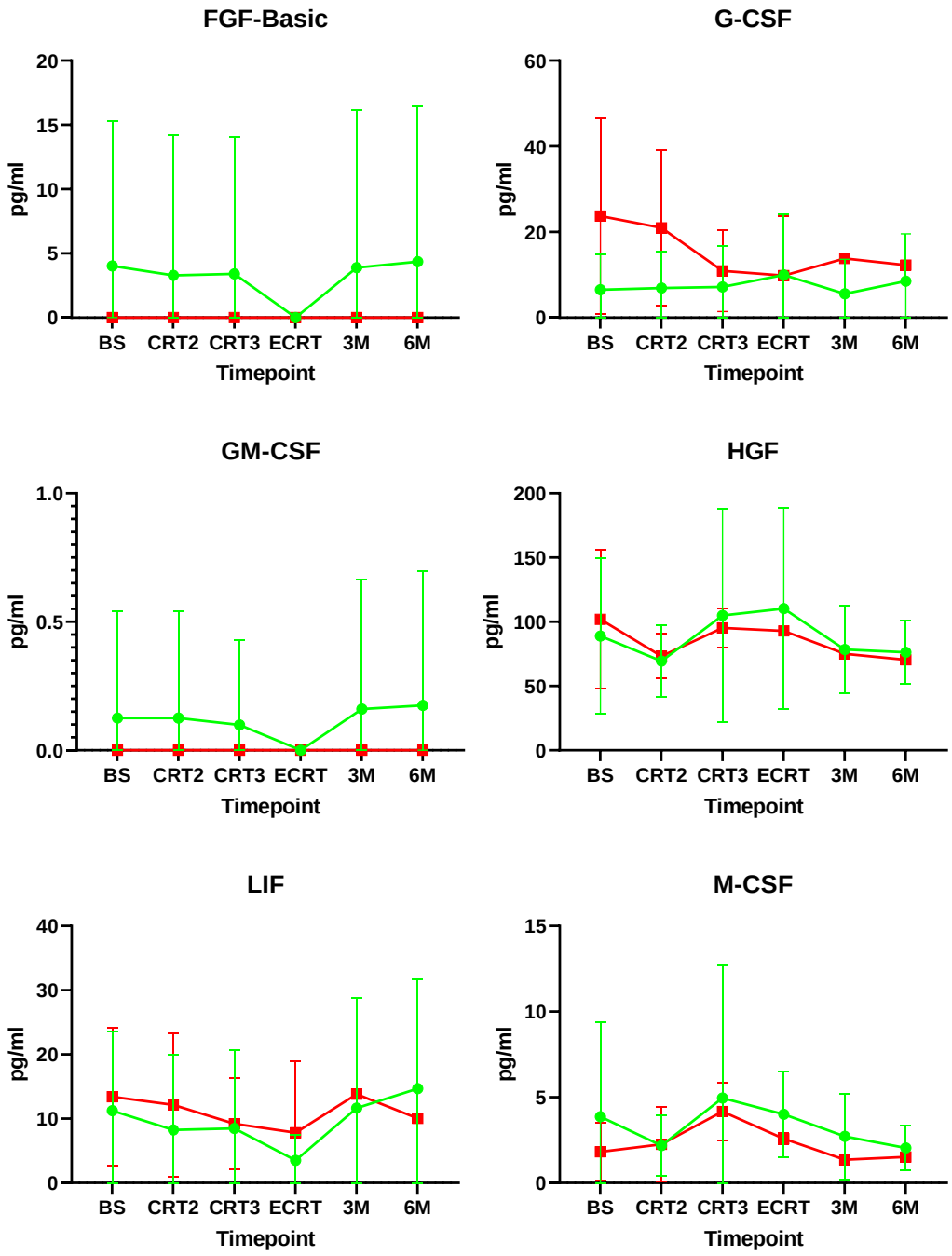


Figure 4.6 Dynamic changes in Growth and Haemopoietic factors FGF-basic, G-CSG, GM-CSF, HGF, LIF, M-CSF for complete response and treatment failure. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

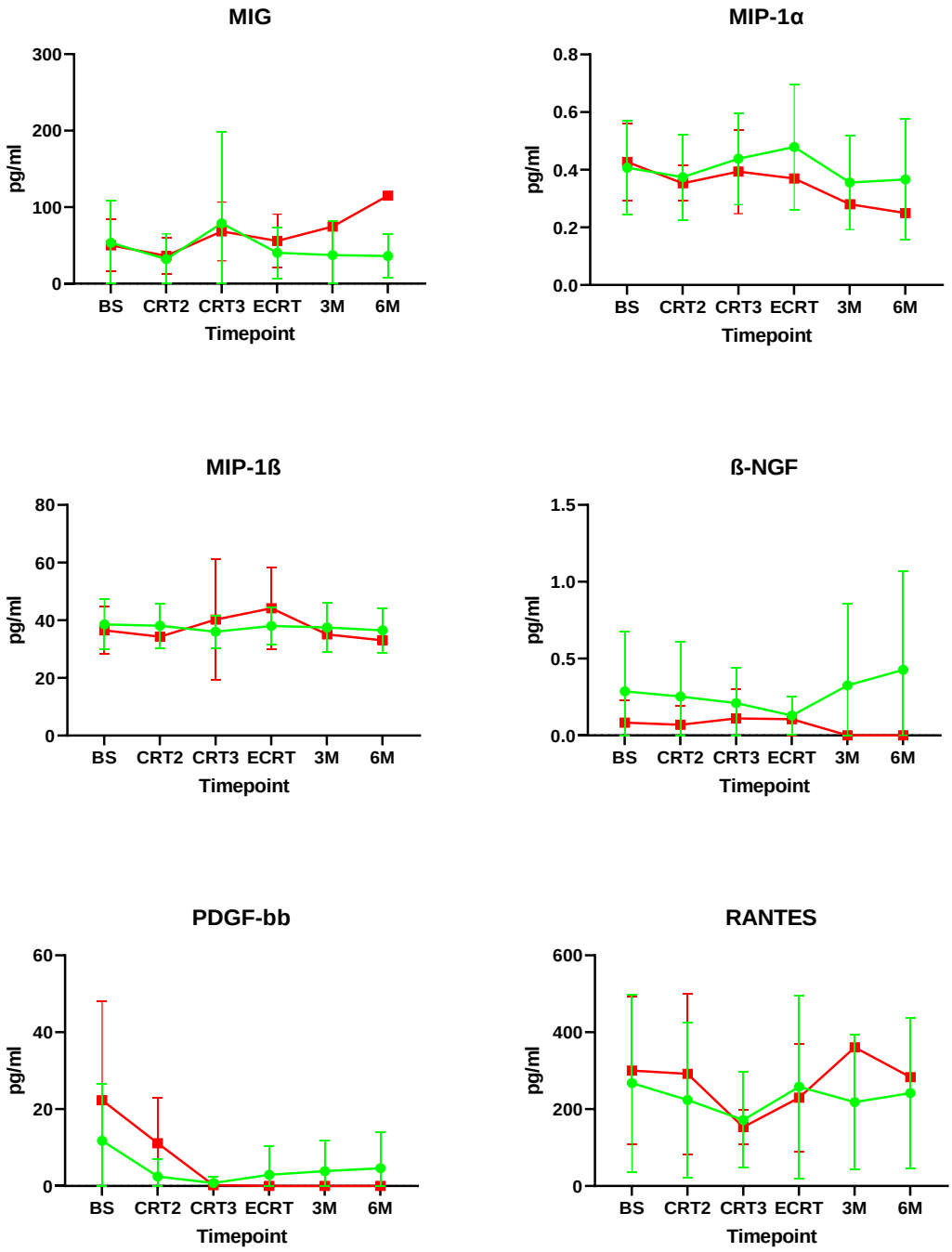


Figure 4.7 Dynamic changes in Growth and Haemopoietic factors PDGF-bb, SCF, SCGF-β, SFD-1α, and β-NGF for complete response and treatment failure. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

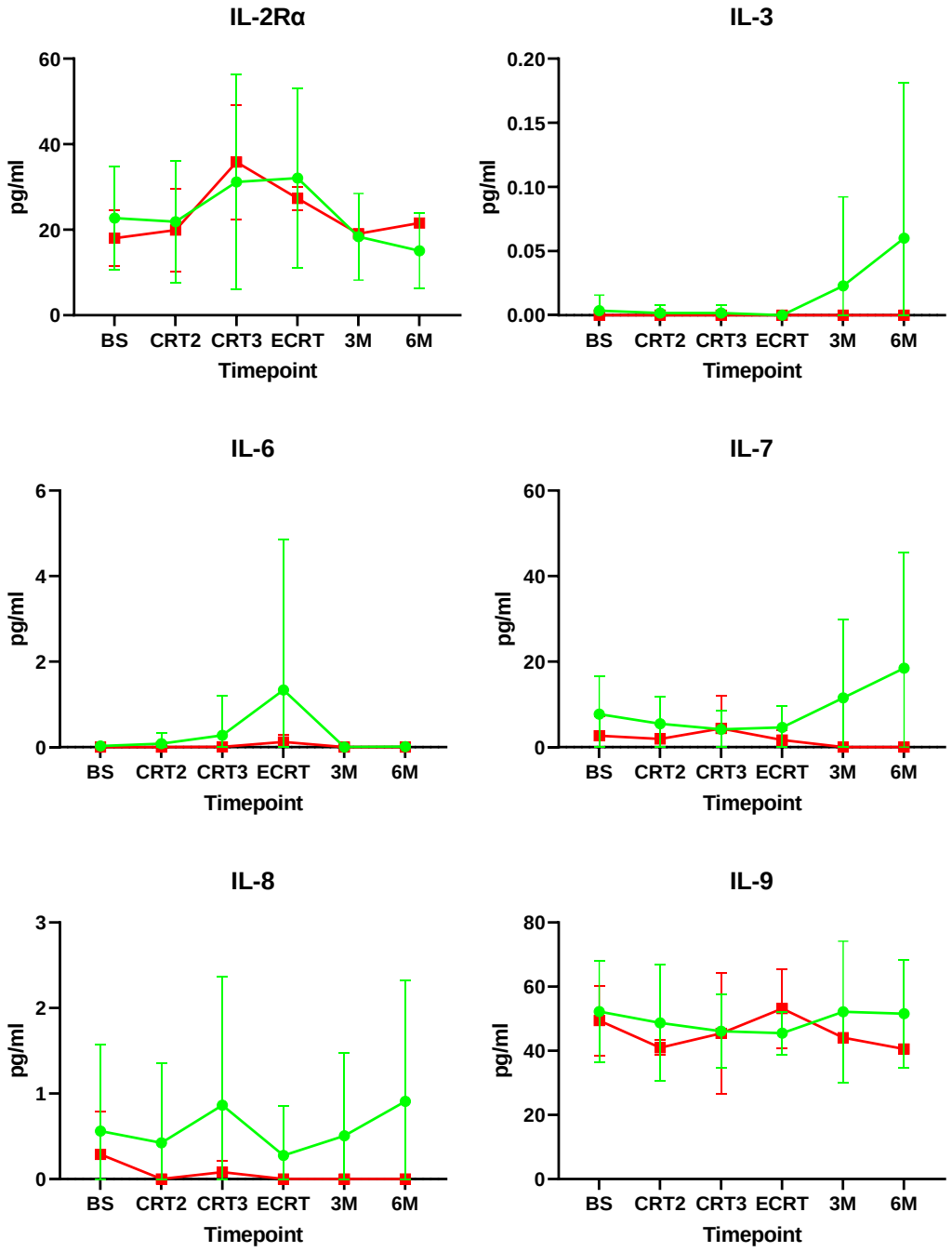


Figure 4.8 Dynamic changes in interleukins IL-2Rα, IL-3, IL-6, IL-7, IL-8, and IL-9 for complete response and treatment failure. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

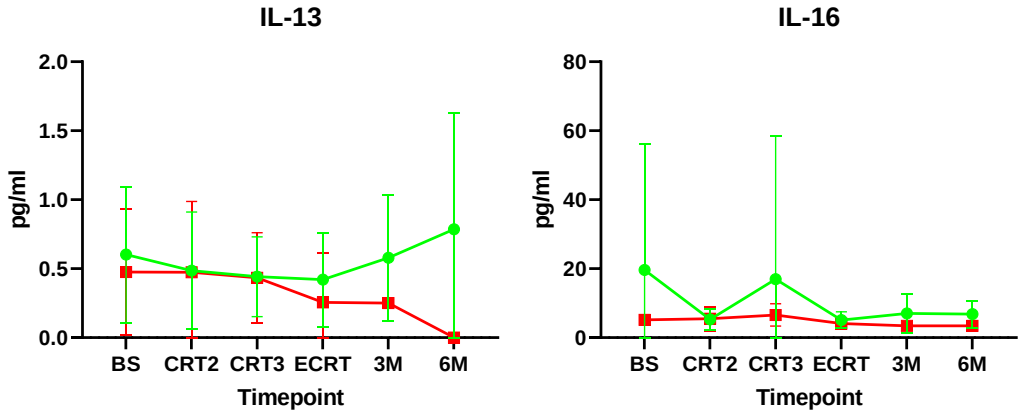


Figure 4.9 Dynamic changes in interleukins IL-13 and IL-16 for complete response and treatment failure. Data is expressed as pg/ml +/-SD. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

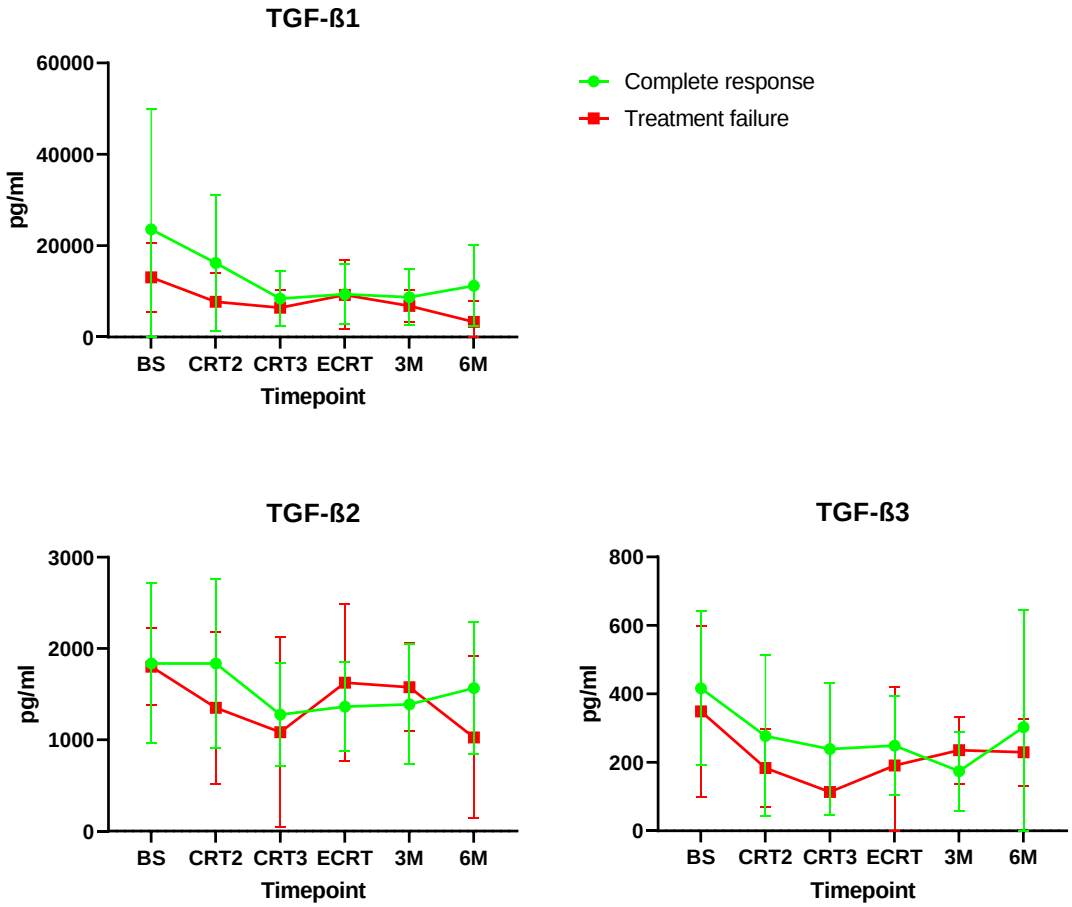


Figure 4.10 Dynamic changes in TGF-β1, TGF-β2, and TGF-β3 for complete response and treatment failure. Data is expressed as pg/ml +/-SD. Data is presented for N=10-12 (CR); N=2-7 (TF), sample availability dependent.

Although there were statistically significant changes in the complete response group from BS to ECRT in IL-1β, IL-17 and MCP-3, the majority of results were <1 pg/ml and changes between timepoints <0.5 pg/m. These were considered negative and are not presented. Figure 4.11 shows within-group comparisons for IL-2Rα and LIF. In the complete response group, there was a trend towards an increase in IL-2Rα from BS to ECRT (22 pg/ml IL-2Rα in complete response at BS to 32 pg/ml at ECRT, p-value 0.22), with a statistically significant decrease in IL-2Rα from ECRT to 6M (32 pg/ml IL-2Rα in complete response at ECRT to 15 pg/ml at 6M, p-value 0.0375). Similar trends were seen in IL-2Rα for the treatment failure group, with an increase from BS to ECRT (18pg/ml IL-2Rα in treatment failure at BS to 27.34 pg/ml at ECRT, p-value 0.16) and a decrease from ECRT to 6M (27.34 pg/ml IL-2Rα in treatment failure at ECRT to 21.57 pg/ml at 6M, stats not performed). Non-parametric tests were used for LIF. There was a statistically significant decrease in LIF from BS to ECRT in the complete response group (median 6.26 pg/ml LIF in complete response at BS to 1.34 pg/ml at ECRT, p-value 0.04), with a trend to increase from ECRT to 6M (median 1.34 pg/ml LIF in complete response to 6.26 pg/ml at 6M, p-value 0.11). There was little change in LIF in the treatment failure group.

Figure 4.12 shows all within-group comparisons for TNF-α. There was a statistically significant drop in TNF-α from BS to ECRT in the complete response group (5.32 pg/ml TNF-α in complete response at BS to 3.06 pg/ml at ECRT, p-value 0.0377), followed by a trend for an increase from ECRT to 6M (3.06 pg/ml TNF-α in complete response at ECRT to 7.1 pg/ml at 6M, p-value 0.082). In the treatment failure group, there was a trend to decrease from BS to ECRT (5.57 pg/ml TNF-α in treatment failure at BS to 1.83 pg/ml at ECRT, p-value 0.091) with a slight decrease from ECRT to 6M (1.83 pg/ml TNF-α in treatment failure at ECRT to 0.6 pg/ml at 6M, stats not performed).

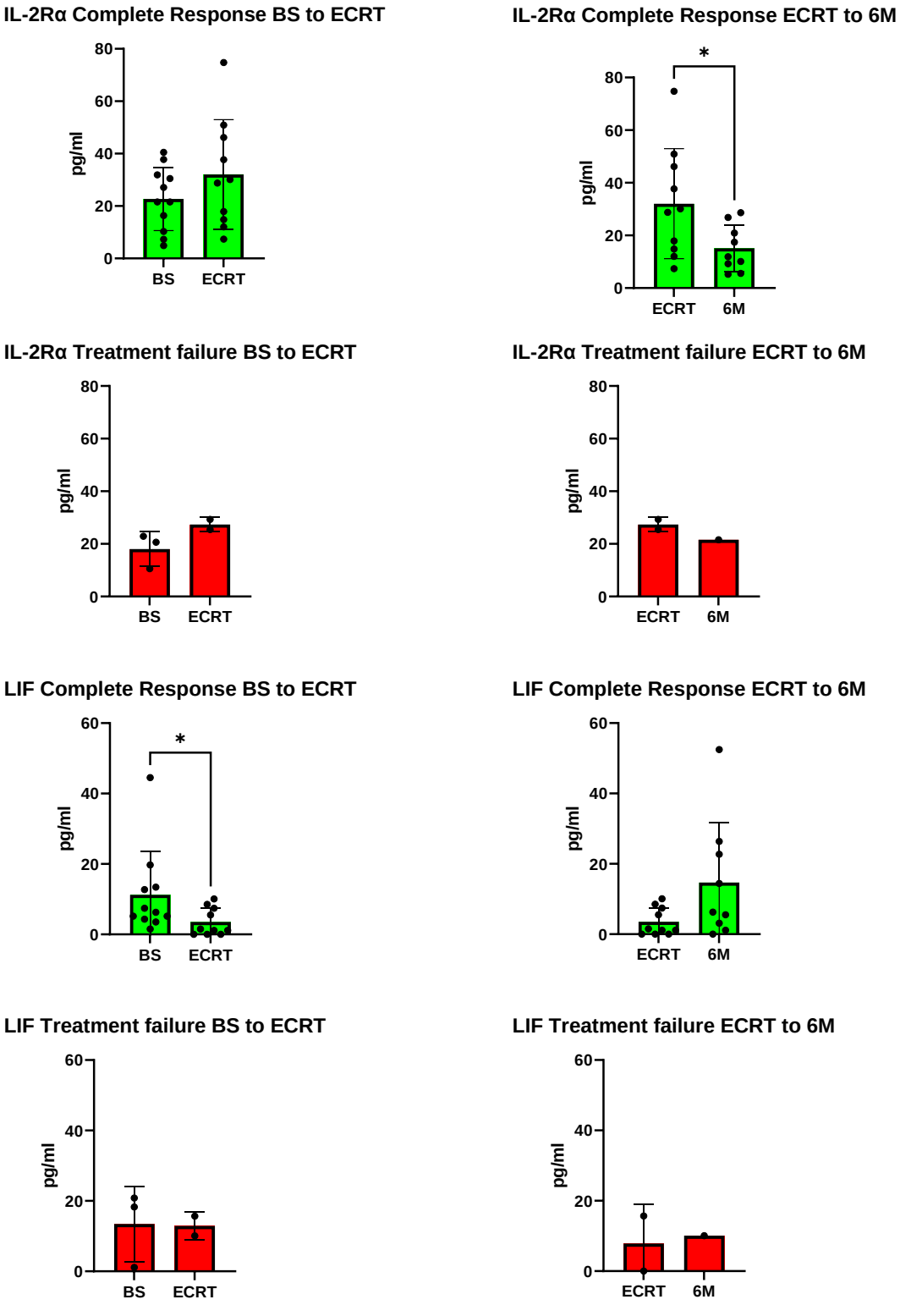


Figure 4.11 Changes in IL-2Rα and LIF from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as pg/ml +/-SD. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

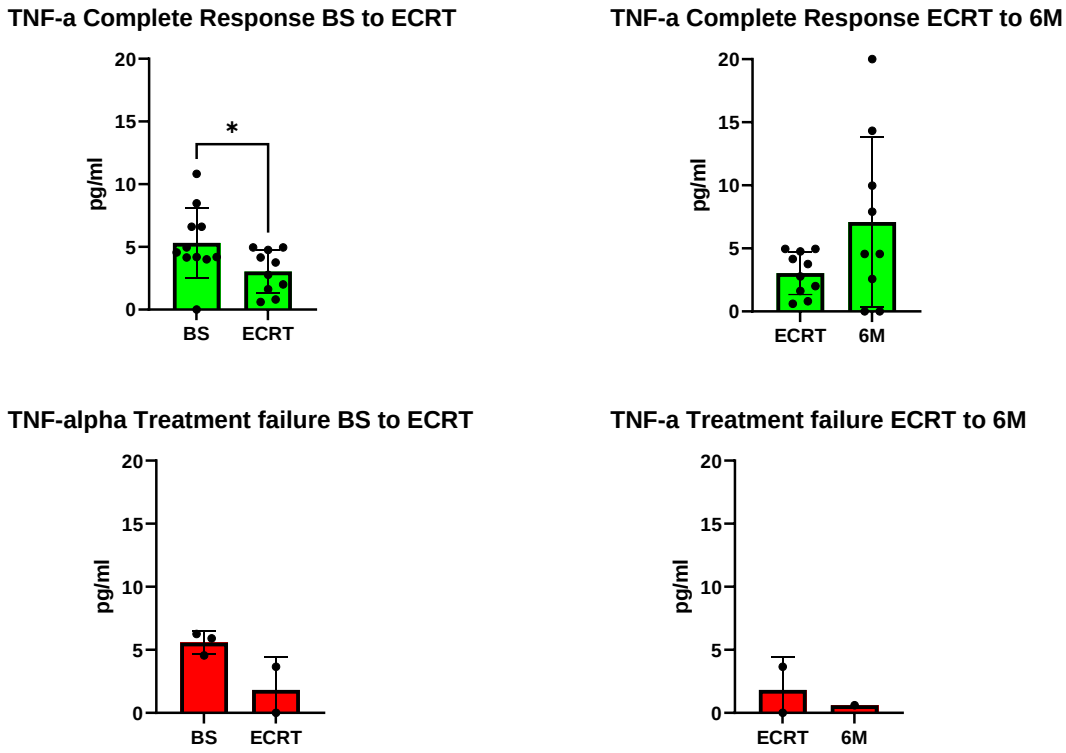


Figure 4.12 Changes in TNF-α and from baseline to end of treatment and end of treatment to 6 months for complete response and treatment failure. Data is expressed as pg/ml +/-SD. Data is presented for N=9-11 (CR); N=1-3 (TF), sample availability dependent.

4.5 Discussion

This chapter used Multiplex ELISA on a large cytokine panel of serial plasma samples to study the soluble markers and dynamics of immune resistance to CRT for locally advanced ASCC. Despite making no adjustments for the multiple comparisons performed, disappointingly, there were no statistically significant differences between patients with complete response and treatment failure. Statistically significant differences were observed from BS to ECRT and ECRT to 6M in the complete response groups. Results were not presented for IL-1β, IL-17 and MCP-3, as many results were below detectable levels, which led to statistics based on inferred values of zero. Given low sample numbers, trends in the treatment failure group from ECRT to 6M should also interpreted with caution. IL-2Rα, LIF, and TNF-α showed significant changes with time, primarily based on the detectable levels.

In this study, IL-2Rα increased during treatment in both groups before returning to baseline at 6 months. IL-2Rα is also known as CD25 and has two forms with distinct functions – membrane-bound and soluble. The membrane-bound forms part of the high-affinity IL-2 receptor complex, promoting the differentiation of T-cells into effector

and memory T-cells. The soluble form measured here is recognised to increase during inflammatory responses, but its role in immunity is not fully understood[220].

IL-2Rα is shed from effector T-cells and Tregs and could function as a sink for IL-2, reducing immune activation. Nickel and colleagues found that soluble CD25 competes with the membrane-bound complex, causing competitive enhancement of $CD25^{high}$ memory/effector and regulatory FOXP3+ subsets[220]. High baseline serum IL-2Rα has been associated with poor prognosis in many solid tumours including HNSCC and oesophageal squamous cancer[221,222]. To my knowledge, there are no studies on the dynamics of IL-2Rα in response to RT in ASCC. In HNSCC, levels of serum IL-2Rα did not change in response to RT[223]. Soluble IL-2Rα has also been shown to inhibit the effects of CTLA-4 and has been suggested as a biomarker of response to CTLA-4 blockade [224,225]. Future ASCC RT-IO trials involving anti-CTLA-4 drugs should assess plasma IL-2Rα measurement as part of the translational analysis[226].

Leukaemia inhibitory factor (LIF) is a member of the IL-6 cytokine family that binds to a heterodimer of LIFR and gp130 on the cell surface. It has a wide variety of physiological and pathological functions. It is overexpressed in many solid cancers and is associated with a poor prognosis[227–229]. LIF has been associated with a polarisation towards an M2-phenotype in tumour-associated macrophages and poor response to PD-1 therapy[230]. Phase I trials using anti-LIF antibodies have shown promise and suggest potential synergy with checkpoint inhibitors[231]. LIF has also been associated with radioresistance in nasopharyngeal cancer, and carbon ion RT has been shown to inhibit LIF-associated proliferation in squamous oesophageal cancer cells[232]. There is no data on the dynamics of LIF in response to CRT in ASCC. Callera and colleagues found no difference in serum level of LIF before, during and after radiotherapy in 48 patients with prostate cancer, although results were not compared between responders and non-responders [224]. In this study, LIF dropped from BS to ECRT in patients with complete response but remained unchanged in those with treatment failure, suggesting a role of radioresistance for LIF in ASCC. Given the early stage of the development of drugs targeting LIF, further measurement is warranted n future ASCC trials but there is currently not enough evidence to support the combination of anti-LIF therapy and CRT in ASCC.

TNF-α is a well-recognised pro-inflammatory cytokine, known to cause cancer cell death since the 1970s[233]. Given that TNF-α is a pro-inflammatory cytokine, one would expect plasma levels to rise during treatment. However, in this study the observed decrease in TNF-α levels during treatment in complete response and treatment failure groups was surprising. In the Callera study discussed earlier, levels of TNF-α were not sensitive to RT, whilst in HNSCC a significant rise in TNF-α in response to RT was seen[234,235]. Following treatment, TNF-α levels recovered in the complete response group compared to the treatment failure group. This could suggest a more pro-inflammatory environment, supporting ongoing ICD after completing CRT in patients

with complete response. Recombinant TNF-α causes high toxicity and has only been used in isolated limb perfusion of advanced melanoma and soft tissue sarcoma[215]. In combination with RT, more sophisticated methods targeting TNF-α via intratumoral injection were safe, avoiding these systematic effects, but did not improve outcomes[236]. All the above suggests that TNF-α is neither a promising biomarker nor a therapeutic target in ASCC.

Although not statistically significant, dynamics of G-CSF were noticeable. In the treatment failure group, baseline levels of G-CSF were higher (median 5.38pg/ml versus 25.66pg/ml, p-value 0.21). G-CSF decreased from BS to ECRT, stabilizing at 6 months. Conversely, G-CSF levels did not change in the complete response group at any timepoint. G-CSF facilitates the production of granulocytes from the bone marrow, of which neutrophils are the most abundant. High baseline neutrophil-lymphocyte ratio (NLR) is a poor prognostic marker in many cancers, including ASCC[21]. There is emerging evidence that low NLR may also predict better response to IO in lung cancer[237]. Neutrophils are part of the innate immune system, and a high NLR is associated with a non-specific acute inflammatory response, associated with higher pro-inflammatory cytokines, peritumoral macrophages and myeloid-derived suppressor cells (MDSCs)[238]. While elevated neutrophil levels can negatively impact the immune response to cancer, they play a crucial role in combating infections; thus, attempts to lower these levels could be potentially life-threatening. G-CSF is administered therapeutically during CRT for certain ASCC patients experiencing severe treatment-induced neutropenia and neutropenic sepsis.

Moreover, there is no literature on high plasma G-CSF levels specifically leading to an increased NLR. NLR data was not investigated in GRECIAN, and we do not know if the higher G-CSF observed was a physiological response to diminished neutrophil counts. Future research could focus on assessing baseline NLR in these GRECIAN patients, examining neutrophil functionality with flow cytometry, and conducting IHC of tumour tissues to assess peritumoral macrophages and MDSCs.

The most disappointing result in this chapter is the lack of difference in TGF-β between groups, particularly TGF-β1. There was no statistically significant difference between the complete response and treatment failure groups. In patients with complete response, there was a trend towards a decrease in TGF-β1 from BS to ECRT, with levels remaining stable from ECRT to 6M. Similar results have been seen in NSCLC. In one study, serum levels of TGF-β1 at baseline before RT were not different between responders and non-responders, but levels were lower in responders following RT[239]. Another study in NSCLC had similar results, finding that patients with decreasing TGF-β1 at the 4th week of RT compared to baseline had a favourable prognosis[240]. The wide confidence intervals in baseline TGF-β1 in GRECIAN make it difficult to know if a similar pattern is occurring here, and measurement of plasma TGF-β is unlikely to be useful in future trials.

One possible reason for the lack of signal in this study could be the measurement of peripheral rather than TME-associated TGF-β. As discussed in the introduction, TGF-β has a dual role in cancer biology, initially acting as a tumour suppressor before switching and suppressing the immune system, facilitating cancer progression. The complex interactions of TGF-β signalling in cancer are regulated by TGF-β Receptor Type I and Type II and phosphorylation of intracellular effector mother against decapentaplegic (SMAD) proteins. SMAD2 and SMAD3 are the main downstream effectors of TGF-β and, despite being structurally very similar, play different and opposing functions in cancer or immune suppression. Additionally, the same SMAD protein can assume multiple roles across different cancers[241]. There is no data on the roles of SMAD proteins in ASCC. TGF-β is secreted in an inactive form, covered in latency-associated peptide that stops binding between the TGF-β contact site and receptor. This activation stage is another possible target for therapy. In melanoma and breast cancer models, Tregs expressing the β8 chain of αvβ8 integrin in the TME are the main cell types that activate TGF-β, and patients that received anti-Itgβ8 antibodies had improved cytotoxic T-cell activation[242]. A better understanding of TGF-β receptors, SMAD proteins, and the importance of different Treg populations in the TME in diagnostic tissue rather than plasma/serum would be a promising avenue for future research in ASCC.

4.5.1 Strengths and weaknesses of this chapter

As with chapter 3, the variety of cytokines measured and the comparison between treatment failure and complete response is a strength of this chapter. Serial cytokine changes in response to CRT have not been measured in ASCC previously. Regardless, the results of this chapter are generally disappointing and do not reveal an obvious target for future RT-IO trials.

There are multiple reasons why the negative results could have occurred. Poor quality of plasma samples and poor laboratory technique are possible but seem unlikely, given that for select cytokines and chemokines, all timepoints were measurable and showed an expected response pattern, such as IL-2Rα. Furthermore, other studies in cytokine dynamics in response to RT have had similar issues. A Norwegian study that investigated the response of inflammatory markers to RT in patients with painful bone metastases using the 27-plex version of the 48-plex Bio-Rad Human Cytokine Panel used in this study also found multiple cytokines below the lower detection limit[243]. Similar results were also found in a study of standard of care long-course CRT in rectal cancer in Glasgow, where levels of many cytokines measured by ELISA were undetectable (personal communication).

Low sample size, particularly for the screening panel, is a weakness of this chapter. No statistical comparisons were possible between treatment failure and complete response at 6M, or ECRT and 6M in treatment failure. This was a difficult but active

decision, given the poor results from the first two plates ran and the substantial costs (~£5000 per plate), meaning that other experiments with potentially interesting results would have to be dropped to continue.

Current Multiplex technology may not measure the appropriate soluble immune resistance mechanisms in this setting. Using different technology, protein differences between complete responders might be detected in plasma. PROphet is a commercially available plasma-based proteomic assay by Oncohost that can predict the benefit of first-line PD-1/PD-L1 inhibition in NSCLC. Using Slow Off-Rate Modified Aptamers, it measures 7000 proteins and assigns a positive or negative score for response to IO[244]. This assay was trialled in HPV-associated cancers, of which 16 had pre-treated metastatic ASCC treated with Bintrafusp alfa, a bifunctional fusion protein targeting TGF-β and PD-L1. Median OS in HPV-associated PROphet-positive vs PROphet-negative groups was 43.6 vs 4.4 months (HR=0.22, 95% CI: 0.08-0.59, p=0.001)[245]. This assay could be used in the GRECIAN samples to assay how these proteins respond to CRT.

4.5.2 Ongoing and future work

As part of a RadNet seed funding grant, multiplex ELISA on different activated and inactivated proteins in the complement system is ongoing. Recent findings indicate that complement is often dysregulated in cancer, contributing to increased tumour cell survival and resistance to immune responses[246,247]. Growing interest exists in exploring complement cleavage products as potential biomarkers detectable from patient plasma. Furthermore, there are a significant number of available complement inhibitors which could be repurposed for cancer treatment once relevant complement biomarkers are identified[248]. An immunosuppressive role for particular complement soluble fragments is now well-documented, including for complement C5a, which mediates MDSC recruitment and attenuated anti-tumour cytotoxic CD8+ T cell responses in CC models[249]. In preclinical studies, targeting complement has emerged as a popular strategy to enhance anti-tumour responses in combination with ICIs. This has led to clinical trials combining anti-C5a receptor (C5aR) and ICIs.[250,251]. Although RT is known to enhance tumour expression of complement products (including C5a), the effects of CRT on complement activation in patient plasma are unexplored[252]. The role of complement in ASCC and whether there is an interaction between complement and other immune resistance markers in this cancer type is also currently unknown.

As discussed in the introduction, cHPV-DNA is burgeoning areas of interest in ASCC and could potentially escalate or de-escalate treatment in future ASCC trials. The relationship between cHPV-DNA dynamics and immune response has never been studied in ASCC. It was intended that cHPV-DNA would be investigated in this chapter. A local assay based on the work of Spindler and colleagues from Aarhus University Hospital, Denmark was in the process of being developed, with the hope that their results would be replicated[91]. However, it has been decided that these samples will be

processed at the ICR using the assay developed by Shree Bhide and colleagues, as this assay has already been used on a subset of the PLATO samples[87].

4.6 Conclusion

The overall aim of this chapter was "To identify pre-CRT and CRT-induced soluble markers of ASCC immune resistance and to correlate these with 6-month treatment failure (circa 25-30%) and complete response (circa 70-75%)."

A Multiplex screening panel investigated 48 cytokines in 14 patients receiving CRT for locally advanced ASCC – 11 with a complete response and 3 with treatment failure at 6 months. A Multiplex TGF-β panel investigated the three isoforms of TGF-β. None of the cytokines investigated showed significant differences between patients with complete response or treatment failure. Although measurement of IL-2Rα and LIF in plasma as part of exploratory translational analysis in future RT-IO ASCC trials is warranted, large screening panels using Multiplex technology should not be a focus for future work in this area.

Chapter 5 Clinical Trial Methodology

5.1 Introduction

This chapter aims to evaluate aspects of clinical methodology relevant to future RT-IO ASCC trials. There are two components of this chapter.

1) A systematic review of current ASCC RT-IO trials
2) The second stage of CORMAC (CORMAC-2). This was published in December 2024 in eClinicalMedicine(https://doi.org/10.1016/j.eclinm.2024.102939). As per the Intellectual Property and Publication Statement at the start of this thesis, my co-author Rebecca Fish was involved in the writing of some of the sections that form part of this chapter.

5.2 Systematic Review

5.2.1 Introduction

Section 1.1.9 in the thesis introduction covers the history of ASCC trials along with the current standard of care. The first and second generations of ASCC trials illustrate the importance of trial design. Apart from ACCORD03, RT dose was not investigated *within* trials but was different *across* trials. Considerable heterogeneity in patient selection, stage of disease, RT technique, RT volumes, and primary outcomes hindered comparisons across these trials of RT doses for the same stage of disease (Table 5.1) As a result, despite extensive high-quality randomised trial data over 30 years, we still do not know the optimal RT dose for different stages of disease, resulting in different practices globally.

Given the heterogeneity described in previous ASCC trials and results from related trials in HNSCC and CC, the design of ASCC RT-IO trials is important. If these trials have negative results, such as in HNSCC, heterogeneity will limit our ability to compare them and draw meaningful conclusions to inform future trials. If similar trials have different results, as in cervical cancer, we may struggle to identify which patients will benefit from the addition of IO. If we are lucky and multiple RT-IO regimens demonstrate benefit, we may end up with several RT-IO regimens recommended for similar ASCC patients without knowing which is optimal, which is the case for current CRT regimens. Given the relative rarity of ASCC compared to HNSCC and CC, there will be fewer trials to identify optimal RT-IO regimens, making it even more important to be able to compare them properly.

In this chapter, a review will summarise and assess the IO regimens, translational analysis plans, and statistical design of RT-IO trials in ASCC. It will elaborate on different aspects of trial design and suggest measures to enable valid trial comparisons in the future. It will summarise the use of any innovative trial designs and evaluate how they could be used for future RT-IO combination trials.

The methods chapter details the search strategy, data extraction, quality assessment, and statistical analysis for the systematic review.

5.2.2 Review question

What are the trial designs, treatment regimens and translational endpoints for RT-IO combination trials in ASCC?

Table 5.1 Radiotherapy characteristics of Phase III randomised trials in ASCC. AP-PA – anterior-posterior and posterio-anterior

Trial	Initial RT dose and technique	Who got RT boost?	Boost dose	Boost technique	Primary outcome	Patient selection	Initial RT field margins	Boost volume
ACT1	45Gy in 20-25F. AP-PA fields	Patients with greater than or equal to 50% response 6 weeks after CRT	15G/6F or 25Gy	Photons, electrons, or brachytherapy	Clinically defined local failure at 6 weeks	All patients including metastatic disease.	Superior margin - mid-pelvic line. Lateral margin – defined by whether lymph nodes were included. Inferior margin - perineum.	Perianal region
EORTC	45Gy in 25F. Three-field or four-field technique	Patients with partial or complete response 6 weeks after CRT	20Gy to partial responders, 15Gy to complete responders	Photons, electrons, or brachytherapy	Clinically defined local failure at 6 weeks	T3-4N0-3 or T1-2N1-3. No metastatic disease	Superior border – promontorium. Lateral border - 1.5cm lateral of the pelvic rim. Inferior border –3cm below the primary tumour.	Original tumour volume with 1cm surrounding tissue. Nodes if involved
RTOG87-04	45Gy-50.4Gy in 25-28F in 1.8Gy/F. AP-PA fields	Patients with positive biopsy 6 weeks after CRT	9Gy/5F	Photons or elections	Local treatment failure defined on biopsy at 4-6 weeks	All localised ASCC. No metastatic disease	Superior border – L4/L5 interspace. Lateral - For N0 disease 1cm lateral to bony pelvis. For N1 disease entire inguinal canal included. Inferior border- included anal	Inguinal nodes if involved and perineum.

Trial	Initial RT dose and technique	Who got RT boost?	Boost dose	Boost technique	Primary outcome	Patient selection	Initial RT field margins	Boost volume
							sphincter and whole perinium.	
RTOG 98-11	45Gy in 25F. AP-PA, three-field, or four-field technique	T3, T4, node positive or T2 with residual disease.	10-14Gy in 2Gy/F	Photons or electrons	Disease-free survival at 5 years	T2-4Nany. No metastatic disease	Include pelvis, anus, perineum, and inguinal nodes. Superior border – L5/S1. Lateral border– determined by bony landmarks. Inferior border - 2.5cm margin around anus and tumour.	Primary tumour volume including involved nodes with a 2-2.5cm margin
ACCORD-03	45Gy in 25F. AP-PA or four-field technique	Patients who responded clinically 3 weeks after CRT.	Standard arm = 15Gy in arm. Experimental arm = 20Gy for patients with complete response (≥ 80% reduction in the primary tumour volume) and 25Gy for minor partial response (<80%)	Photons, electrons, or brachytherapy	Colostomy-free survival at 2 years	T>4cmNany. No metastatic disease	Superior – L5/S. Lateral – Pelvic brim or wider if inguinal areas were included. Inferior – Perianal region	Anorectal region

Trial	Initial RT dose and technique	Who got RT boost?	Boost dose	Boost technique	Primary outcome	Patient selection	Initial RT field margins	Boost volume
ACT2	36Gy in 17F. AP-PA fields	All patients	19.8Gy in 11F	Photons or brachytherapy	Clinical complete response at 6 months	All localised ASCC. No metastatic disease	Superior – 2cm above bottom of SI joint. Lateral – Lateral to femoral heads to cover inguinal nodes. Inferior – 3cm below anal margin (canal only tumours) or 3cm below most inferior extent of tumours (anal margin tumours)	Primary tumour and whole anal canal with 3cm margin around all macroscopic tumours

5.2.3 Results

Eleven trials were included in the review, see Figure 5.1 for the PRISMA diagram. Table 5.2 lists each of the included trials. All trials were registered with clinicaltrials.gov, and two had protocols attached to this record. Two trials had protocol papers published in academic journals, one full protocol was sent to the authors, with two other investigators providing details of translational endpoints and trial schemas without sending the complete protocol. Commercial sensitivities limited data extraction and analysis for at least three trials.

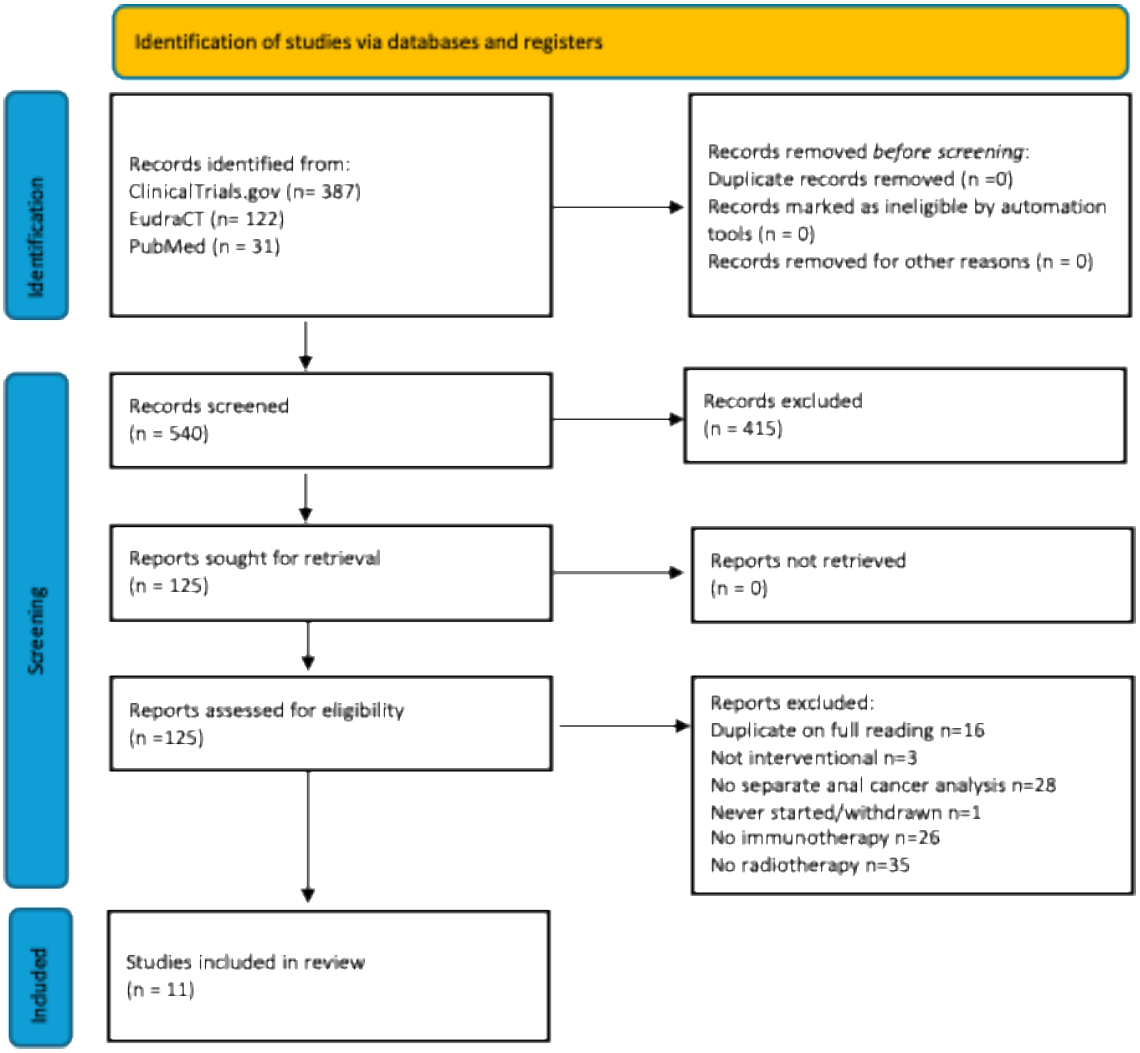


Figure 5.1 PRISMA Flow Diagram for RT-IO ASCC Systematic Review

Table 5.2 Trial design characteristics of RT-IO Combination Trials for ASCC. AEs – Adverse events, cCR – Clinical Complete response, CFS – Colostomy Free Survival, DFS – Disease-Free Survival, ORR – Objective Response Rate, PFS- Progression Free Survival, PROs –Patient Reported Outcomes

Study	Protocol or publication available?	Stage of disease	Primary Endpoints	Secondary Endpoints	Location	Phase	Number of arms	Randomised?	Biomarker selection	Statistical design	(Proposed) sample size	Finished, recruiting, or setting up?
A Phase I/II Evaluation of ADXS11-001, Mitomycin, 5-fluorouracil (5-FU) and IMRT for Anal Cancer (BrUOG276) (NCT01671488)	Yes	Locally advanced disease	Safety and 6-month cCR	PFS, OS; will correlate HPV subtype with all other endpoints	USA	1/2	1	No	No	Not stated	11	Finished, pilot data published to be used for upcoming larger trial
Nivolumab After Combined Modality Therapy in Treating Patients with High Risk Stage II-IIIB Anal Cancer (NCI-EA2165) (NCT03233711)	No, additional information provided	Locally advanced disease	5-year DFS	ORS, toxicity, CFS, OS (up to 5years)	USA	3	2	Yes	No	Not stated	344	Active but not recruiting

Study	Protocol or publication available?	Stage of disease	Primary Endpoints	Secondary Endpoints	Location	Phase	Number of arms	Randomised?	Biomarker selection	Statistical design	(Proposed) sample size	Finished, recruiting, or setting up?
Prebiotics and Probiotics During Definitive Treatment with Chemotherapy-radiotherapy SCC of the Anal Canal (BISQUIT) (NCT03870607)	Yes	≥ T2N0M0	6–8-week cCR	Metabolic response measured by PET-CT, 6-month cCR, 1-year PFS and CFS, toxicity, HPV genotyping, variations in systemic immune parameters	Brazil	2	2	Yes	No	Unclear – comparative analysis but single arm design approach used for sample size calculation	75	Recruiting
Phase 1B/II Trial of Checkpoint Inhibitor (Pembrolizumab an Anti PD-1 Antibody) Plus Standard IMRT in HPV Induced Stage III/IV Carcinoma of Anus (CORINTH) (NCT04046133)	Yes	Locally advanced disease	Grade 3/4 toxicity up to 12 months	Adherence to protocol in terms of treatment, recruitment, retention, study eligibility. Clinical Response. Radiological response. PROs	UK & Norway	1b/2	3	Yes	No	None detailed. Early stopping rules for safety provided	50	Recruiting

Study	Protocol or publication available?	Stage of disease	Primary Endpoints	Secondary Endpoints	Location	Phase	Number of arms	Randomised?	Biomarker selection	Statistical design	(Proposed) sample size	Finished, recruiting, or setting up?
Radiochemotherapy +/- Durvalumab for Locally-advanced Anal Carcinoma. A Multicentre, Randomised, Phase II Trial of the German Anal Cancer Study Group (RADIANCE) (NCT04230759)	Yes	Locally advanced disease	3-year DFS	Acute toxicity, complete remission at 6 months, OS, CFS, incidence of recurrence, QoL	Germany	2	2	Yes	No	Log-rank test of equal exponential survival	178	Recruiting
Anti-PD-1 and mDCF Followed by Chemoradiotherapy in Patients with Stage III Squamous Cell Anal Carcinoma (INTERACT-ION) (NCT04719988)	Yes	Locally advanced disease	10-month cCR	Major pathological response (complete/near-complete response) and biological CR (HPV ctDNA negative). ORR, OS, PFS, RFS, HRQoL and safety	France	2	2	No	No	A'Hern exact design	55	Active, not recruiting

Study	Protocol or publication available?	Stage of disease	Primary Endpoints	Secondary Endpoints	Location	Phase	Number of arms	Randomised?	Biomarker selection	Statistical design	(Proposed) sample size	Finished, recruiting, or setting up?
Combination of Spartalizumab, mDCF and Radiotherapy in Patients with Metastatic Squamous Cell Anal Carcinoma (SPARTANA) (NCT04894370)	No, additional information provided	Metastatic or recurrent	1-year PFS	ORR, OS, median PFS	France	2a	1	Not clear	No	Not stated	47	Recruiting
Therapy Adapted for High Risk and Low Risk HIV-Associated Anal Cancer (NCT04929028)	No	Locally advanced for immunotherapy arm	Safety of reduced IMRT in low-risk disease, safety of nivolumab in high-risk disease– Grade 3/4 toxicity	DFS at 2 years, disease control rate, change in CD4+ count, change in HIV viral load, change in cART adherence	USA	2	2	No	HIV positive only	Not stated	53	Recruiting

Study	Protocol or publication available?	Stage of disease	Primary Endpoints	Secondary Endpoints	Location	Phase	Number of arms	Randomised?	Biomarker selection	Statistical design	(Proposed) sample size	Finished, recruiting, or setting up?
Neoadjuvant PD-1 Blockade Combined with Chemotherapy Followed by Concurrent Immunoradiotherapy for Locally Advanced Anal Canal Squamous Carcinoma Patients (NCT05060471)	Yes	Locally advanced disease	3-month cCR	6-month cCR 6, acute and late toxicity, colostomy rate, local recurrence rate, distant metastasis rate, PFS, OS	China	Cohort study	1	No	No	Observational cohort	27	Early results published, trial still recruiting
Chemoradiotherapy Combined with or Without PD-1 Blockade in Anal Canal Squamous Carcinoma Patients (NCT05374252)	No	Locally advanced disease	3-year PFS, 3-year OS, 6-month cCR	Acute and late toxicity, colostomy rate, local recurrence rate, metastasis rate	China	3	2	Yes	No	Not stated	102	Recruiting
Atezolizumab plus tiragolumab in combination with chemoradiotherapy in localised squamous cell carcinoma of the anal canal: TIRANUS (NCT05661188)	No	Local disease	6-month cCR	AEs, Locoregional failure rate, 5-year CFS, DFS and OS. HRQoL, safety.	Spain	2	1	No	No	Not stated	45	Recruiting

5.2.3.1 Study Results

As expected, only two trials had published preliminary results. BrUOG276 used a Listeria-based therapeutic cancer vaccine targeting HPV-16 E7, with nine patients completing treatment, of whom eight were progression-free at the time of analysis, with a median follow-up of 42 months and a good safety profile reported[253]. NCT05060471 has published safety and biomarker analysis from the first five patients recruited, with all patients alive and disease-free at the time of analysis with 21.8 months median follow-up and a good toxicity profile[254]. NCI-EA2165 has finished recruitment but is awaiting results. INTERACT-ION is active but not recruiting. All other trials are currently recruiting.

5.2.3.2 Trial design

Statistical design information was limited across the trials, with no information available for seven trials. One study had a mismatch between design and analysis, one was an observational cohort, one used the A'Hern single-arm design, and one was a comparative phase II design to compare survival. No trials incorporated biomarker selection, except for NCT04929028 for HIV-positive patients only. All trials that met the inclusion criteria exclusively investigated ASCC. Apart from SPARTANA, which investigated metastatic or recurrent disease, all trials were for patients with local disease. Five (45%) trials were randomised, although no clear information regarding the intention to compare arms was provided for four of these five.

CORINTH used a planned sequential approach to safety evaluation by recruiting sequentially into two, Phase Ib cohorts. Early stopping rules were provided for safety; no statistical detail was provided regarding the design and decision-making. If early safety data from the first cohort of six patients receiving pembrolizumab at week 4 of CRT is satisfactory, the next cohort will receive it from week 1. Following this, 32 patients will be recruited at the most appropriate dosing regimen. INTERACT-ION used a novel design feature for an involved-node RT approach. All patients receive 2 months of chemo-immunotherapy, and those who do not have progressive disease at this point receive a further 1 month of chemo-immunotherapy. Patients with a clinical objective response in initially involved lymph nodes receive 45Gy instead of their standard of care of 59.4Gy to these nodes. Clinical objective response was defined as radiological response ≥30% by RECIST, pathological complete or near complete response (viable tumour cells/tumour bed at biopsy ≤10%), and biological complete response (no residual circulating HPV DNA (cHPV-DNA))[255]. As shown in Table 5.2, a wide variety of primary and secondary endpoints were used that likely reflect the different Phases of each clinical trial, with Phase I trials primarily interested in safety and Phase III trials DFS and PFS. None of the trials had the same primary endpoint, and none measured this at the same time. Overall survival is the only endpoint that was assessed across all trials.

5.2.3.3 Patient selection, chemotherapy regimens, and radiotherapy regimens

Table 5.3 shows the patient selection criteria, and Table 5.4 shows the chemotherapy and radiotherapy regimens. Of the nine trials that used concurrent chemotherapy, four allowed fluoropyrimidine choice, and five allowed only 5-FU. Five used MMC only, one used cisplatin (CisP) only, and three allowed choice between MMC or CisP. Within MMC/CisP dosing, three trials had one dose, three trials had two doses, one trial had one or two doses, and one was unclear.

Within local disease trials, all but two (NCT04929028 and TIRANUS) investigated locally advanced disease only; NCT04929028 included HIV+ early-stage disease, but only patients with locally advanced disease received IO as part of this trial. Five different definitions of locally advanced disease were used across the eight trials. BrUOG276 and TIRANUS used version 7 of the AJCC/UICC staging, while all others used version 8.

All trials for local disease used radical fractionated radiotherapy, with 2 using chemo-immunotherapy before CRT. The variability in doses to the primary tumour, involved lymph nodes, and elective lymph nodes reflects the national guidelines where each trial was conducted. SPARTANA used a single 8Gy dose of radiotherapy to stimulate an immune response to a metastatic lesion.

5.2.3.4 Immunotherapy regimens

Table 5.4 shows the variation in the timing of IO with RT. One trial used neoadjuvant IO only, one neoadjuvant and concurrent IO, and two neoadjuvant and adjuvant IO. Three trials used concurrent and adjuvant IO, and two adjuvant IO only. Two trials used neoadjuvant, concurrent and adjuvant IO. Of the 11 trials investigating immunomodulatory regimens, nine investigated checkpoint inhibitors (seven PD-1 antibodies, one PD-L1 antibody, one a combination of PD-L1 with a TIGIT antibody), one a therapeutic cancer vaccine, and one pre/probiotics. Figure 5.2 demonstrates the heterogeneity for all trials in the treatment modality regimens, including how long each regimen was given.

Table 5.3 Treatment regimens and patient selection criteria for RT-IO Combinations trials for ASCC. *Dictated by response assessment. 5 ** Based in AJCC v7 which had N1-3, now categorised as N1a, N1b, N1c

Study	Patient selection criteria							
	T1N0M0	T1N1M0	T2N0M0	T2N1M0	T3N0M0	T3N1M0	T4N0M0	T4N1M0
A Phase I/II Evaluation of ADXS11-001, Mitomycin, 5-fluorouracil (5-FU) and IMRT for Anal Cancer (BrUOG276)		✓**	✓	✓**	✓	✓**	✓	✓
Nivolumab After Combined Modality Therapy in Treating Patients with High Risk Stage II-IIIB Anal Cancer (NCI-EA2165)				✓	✓	✓	✓	✓
Prebiotics and Probiotics During Definitive Treatment with Chemotherapy-radiotherapy SCC of the Anal Canal (BISQUIT)			✓	✓	✓	✓	✓	✓
Phase 1B/II Trial of Checkpoint Inhibitor (Pembrolizumab an Anti PD-1 Antibody) Plus Standard IMRT in HPV Induced Stage III/IV Carcinoma of Anus (CORINTH)					✓	✓	✓	✓

Study	Patient selection criteria							
	T1N0M0	**T1N1M0**	**T2N0M0**	**T2N1M0**	**T3N0M0**	**T3N1M0**	**T4N0M0**	**T4N1M0**
Radiochemotherapy +/- Durvalumab for Locally-advanced Anal Carcinoma. A Multicentre, Randomised, Phase II Trial of the German Anal Cancer Study Group (RADIANCE)				✓, only if >4cm	✓	✓	✓	✓
Anti-PD-1 and mDCF Followed by Chemoradiotherapy in Patients with Stage III Squamous Cell Anal Carcinoma (INTERACT-ION)		✓		✓		✓	✓	✓
Combination of Spartalizumab, mDCF and Radiotherapy in Patients with Metastatic Squamous Cell Anal Carcinoma (SPARTANA)	Metastatic or recurrent disease							
Therapy Adapted for High Risk and Low Risk HIV-Associated Anal Cancer (NCT04929028)				✓	✓	✓	✓	✓
Neoadjuvant PD-1 Blockade Combined with Chemotherapy Followed by Concurrent Immunoradiotherapy for Locally Advanced Anal Canal Squamous Carcinoma Patients (NCT0506047)		✓	✓	✓	✓	✓	✓	✓

Study	Patient selection criteria							
	T1N0M0	**T1N1M0**	**T2N0M0**	**T2N1M0**	**T3N0M0**	**T3N1M0**	**T4N0M0**	**T4N1M0**
Chemoradiotherapy Combined with or Without PD-1 Blockade in Anal Canal Squamous Carcinoma Patients (NCT05374252)		✓	✓	✓	✓	✓	✓	✓
Atezolizumab plus tiragolumab in combination with chemoradiotherapy in localised squamous cell carcinoma of the anal canal: (TIRANUS)		✓**	✓	✓*	✓	✓**	✓	✓

Table 5.4 Treatment regimens for RT-IO Combinations trials for ASCC 5-FU = 5-fluorouracil, Cap = Capecitabine, CisP = Cisplatin, MMC = Mitomycin C

Study	Concurrent chemotherapy regime	Radiotherapy regimen			Type of Immunotherapy	Timing of immunotherapy		
		Dose to Primary Tumour	Dose to involved nodes	Elective node dose		Neoadjuvant	Concurrent	Adjuvant
A Phase I/II Evaluation of ADXS11-001, Mitomycin, 5-fluorouracil (5-FU) and IMRT for Anal Cancer (BrUOG276)	5-FU/MMC. 2 doses of MMC	54Gy/30# but can give 59.4Gy/33# sequential boost at investigators discretion	54Gy/30#	45Gy/30#	Tumour vaccine	✓		✓
Nivolumab After Combined Modality Therapy in Treating Patients with High Risk Stage II-IIIB Anal Cancer (NCI-EA2165)	5-FU/MMC, Cap/MMC or 5-FU/CisP. 2 doses of CisP/MMC	Not stated	Not stated	Not stated	PD-1 antibody			✓
Prebiotics and Probiotics During Definitive Treatment with Chemotherapy-radiotherapy SCC of the Anal Canal (BISQUIT)	5-FU/MMC, Cap/MMC or 5-FU/CisP. 2 doses of CisP/MMC	Not stated	Not stated	Not stated	Prebiotics and Probiotics	✓	✓	✓
Phase 1B/II Trial of Checkpoint Inhibitor	5-FU/MMC or Cap/MMC. CisP	53.2Gy/28#	50.4Gy/28#	40Gy/28#	PD-1 antibody		✓	✓

Study	Concurrent chemotherapy regime	Radiotherapy regimen			Type of Immunotherapy	Timing of immunotherapy		
		Dose to Primary Tumour	Dose to involved nodes	Elective node dose		Neoadjuvant	Concurrent	Adjuvant
(Pembrolizumab an Anti PD-1 Antibody) Plus Standard IMRT in HPV Induced Stage III/IV Carcinoma of Anus (CORINTH)	can be used at investigators discrepancy. 1 dose of CisP/MMC							
Radiochemotherapy +/- Durvalumab for Locally-advanced Anal Carcinoma. A Multicentre, Randomised, Phase II Trial of the German Anal Cancer Study Group (RADIANCE)	5-FU/MMC. 1 dose of MMC.	53.2Gy/28# for smaller tumours, 58.9Gy/30# for larger tumours	50.4Gy/28#	40Gy/28#	PD-L1 antibody	✓	✓	✓
Anti-PD-1 and mDCF Followed by Chemoradiotherapy in Patients with Stage III Squamous Cell Anal Carcinoma (INTERACT-ION)	5-FU/MMC. 1 or 2 doses of MMC	59.4Gy/33#	59.4Gy/33# or 45Gy/33#*	45Gy/33#	PD-1 antibody	✓		✓
Combination of Spartalizumab, mDCF and Radiotherapy in Patients	Not applicable	8Gy/1# to target lesion	Not applicable	Not applicable	PD-1 antibody	✓		

Study	Concurrent chemotherapy regime	Radiotherapy regimen			Type of Immunotherapy	Timing of immunotherapy		
		Dose to Primary Tumour	Dose to involved nodes	Elective node dose		Neoadjuvant	Concurrent	Adjuvant
with Metastatic Squamous Cell Anal Carcinoma (SPARTANA)								
Therapy Adapted for High Risk and Low Risk HIV-Associated Anal Cancer (NCT04929028)	5-FU/MMC or Cap/MMC. 1 dose of MMC	Not stated	Not stated	Not stated	PD-1 antibody			✓
Neoadjuvant PD-1 Blockade Combined with Chemotherapy Followed by Concurrent Immunoradiotherapy for Locally Advanced Anal Canal Squamous Carcinoma Patients (NCT0506047)	None	50Gy/25#	50Gy/25#	42.5Gy/25 to inguinal region, 45Gy/25# to all others	PD-1 antibody	✓	✓	
Chemoradiotherapy Combined with or Without PD-1 Blockade in Anal Canal Squamous Carcinoma Patients (NCT05374252)	5-FU/MMC. Not clear on doses of MMC	Not stated	Not stated	Not stated	PD-1 antibody		✓	✓

Study	Concurrent chemotherapy regime	Radiotherapy regimen			Type of Immunotherapy	Timing of immunotherapy		
		Dose to Primary Tumour	Dose to involved nodes	Elective node dose		Neoadjuvant	Concurrent	Adjuvant
Atezolizumab plus tiragolumab in combination with chemoradiotherapy in localised squamous cell carcinoma of the anal canal: (TIRANUS)	5-FU/CisP. 2 doses of CisP.	54Gy/30#	Not stated	Not stated	PD-L1 antibody and TIGIT antibody		✓	✓

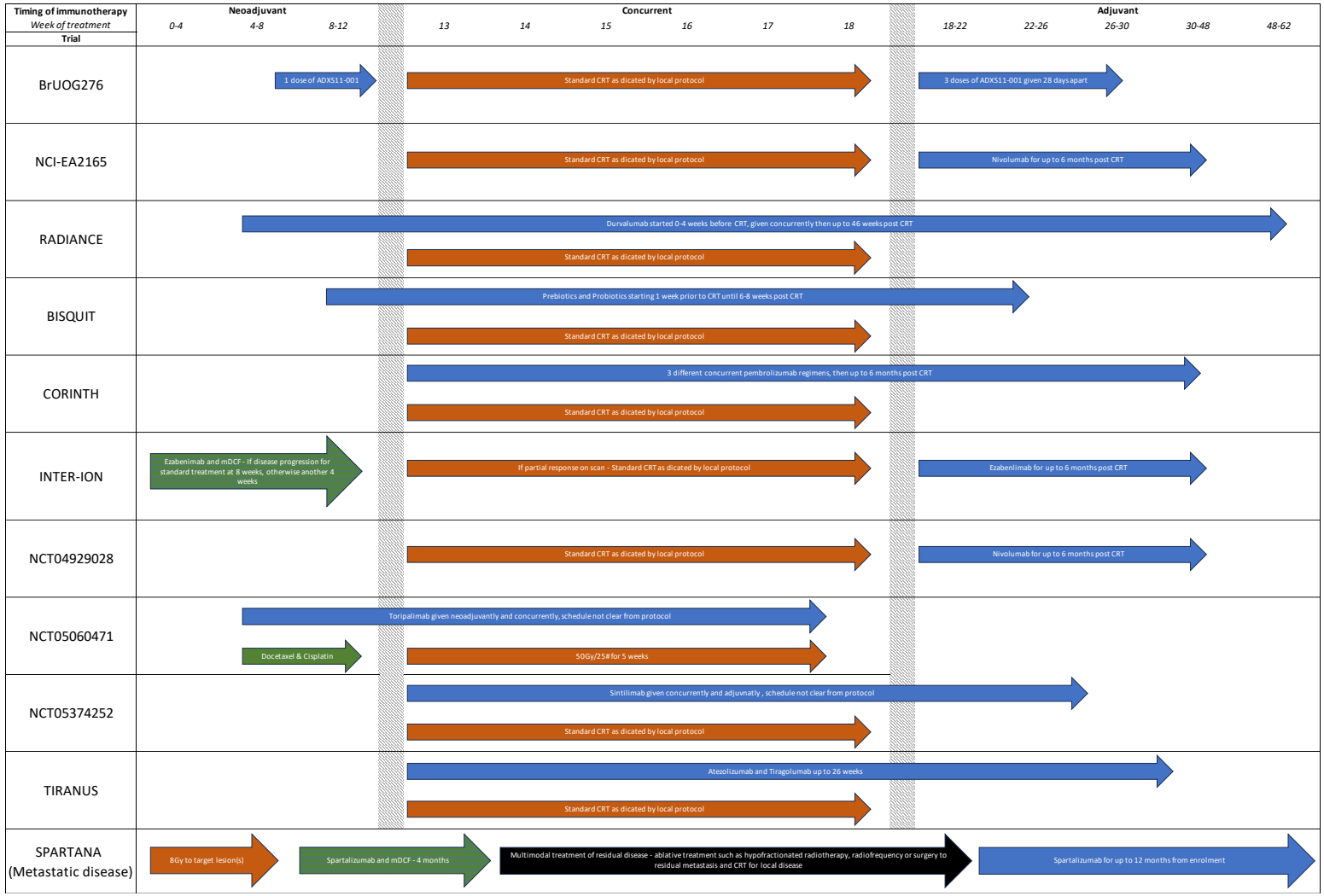
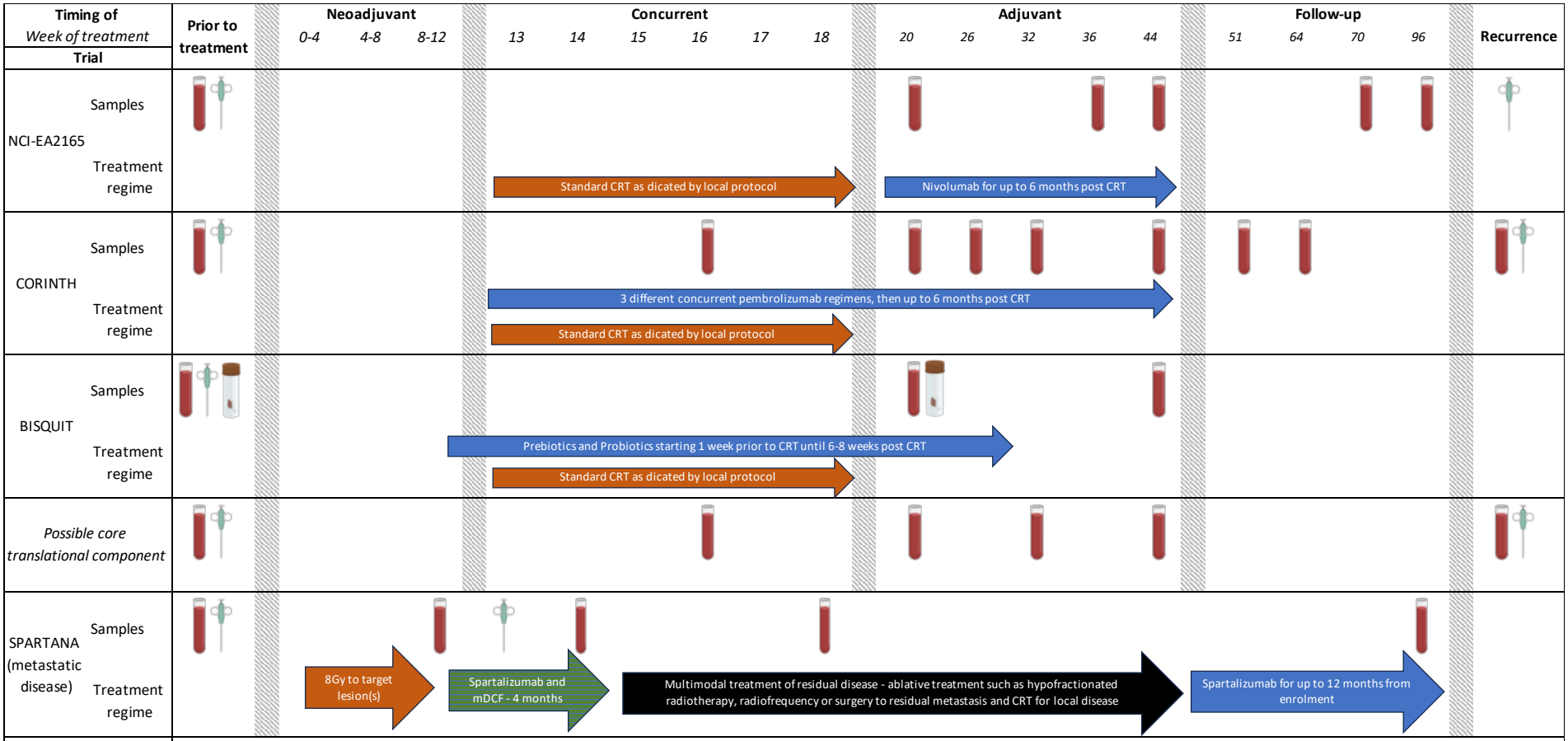


Figure 5.2 All treatment modalities for RT-IO ASCC trials, including length of each treatment modality

5.2.3.5 Translational analysis

Data outlining translational analyses was available for six trials, with timepoint data available for four of these. Figure 5.3 shows all translational trials that had available data on timepoints. All trials take blood and tissue for research purposes before starting treatment, with two taking blood and/or tissue at recurrence. Planned analysis differs across trials, with at least five investigating cHPV-DNA and at least four trials collecting peripheral blood mononuclear cells. Although not stated in all trials, cHPV-DNA is being assessed for its prognostic value before treatment, as a tool to assess response to treatment, or as a biomarker for disease recurrence. Peripheral blood mononuclear cells are being collected for various immune-based techniques, such as immunophenotyping, but further details were not available for any of the trials.



INTER-ION	Extensive translational samples being performed including blood and tissue but details not available due to commercial sensitivities (authors reply)
NCT04929028	Translational tissue samples but imepoints not available.
NCT05060471	Preliminary tissue sample results published
NCT05374252	None reported/performed
BrUOG276	None reported/performed
RADIANCE	Extensive tissue and blood translational samples being performed but details not available due to commercial sensitivities (authors reply)
TIRANUS	Extensive tissue and blood translational samples being performed but details not available, no author reply

Figure 5.3 Translational analysis plan for RT-IO ASCC studies and possible core translational component

5.2.4 Discussion

In this review, 11 trials that combined RT and IO for the treatment of ASCC were identified. All possible combinations of neoadjuvant, concurrent, and adjuvant IO were investigated, and multiple types of IO were used. Apart from a small amount of preliminary data from two trials, no trial has reported results.

5.2.4.1 Immunotherapy regimens

As expected, most trials investigated drugs that target PD-1/PD-L1, with a therapeutic vaccine, an anti-TIGIT antibody and pre/probiotics also investigated. Although different types of IO will interact with RT differently, even within PD-1/PD-L1 inhibitors, the exact mechanism may be important. Differing results from CALLA and KEYNOTE-A18 may be due to the better efficacy of PD-1 compared to PD-L1 inhibitors in CC[256]. However, this data is from metastatic CC in patients not treated with RT. Unfortunately, this difference in the outcomes between CALLA and KEYNOTE-A18 cannot be entirely understood due to a lack of translational analysis in KEYNOTE-A18[257]. Of the two approved RT-IO combinations in the UK, one is nivolumab, a PD-1 inhibitor for oesophageal cancer due to the results of the Checkmate577 trial, and the other is durvalumab, a PD-L1 inhibitor for NSCLC due to the PACIFIC trial. This demonstrates the limitations of extrapolating optimal RT-IO combinations from other tumour sites and data from the same tumour site in a different setting, emphasising the importance of translational analysis.

Pre-clinical data supports the integration of neoadjuvant, concurrent, and adjuvant IO with RT [135,258,259]. Interpreting this data for optimal timing of IO with RT for trial design is challenging. The PACIFIC and Checkmate577 trials used adjuvant-only IO, with PACIFIC after CRT only and Checkmate577 after CRT, followed by surgical resection in oesophageal cancer with residual disease. In contrast, the substantial lymphopenia caused by CRT for HNSCC could explain why concurrent IO, as given in JAVELIN100 and Keynote412, did not show benefit. Both these trials also gave adjuvant IO, and there are currently no results from Phase III trials for adjuvant IO only in HNSCC. Even within adjuvant IO, timing may be important - patients in the PACIFIC trial who started IO within 14 days after CRT had better outcomes than those who started >14 days[260]. All these trials used checkpoint inhibitors, and other types of IO are currently investigated across tumour types, including ASCC. Optimal timing with RT may depend on the type of IO being used. This review found significant diversity in IO timing for RT-IO ASCC trials - all possible combinations of neoadjuvant, concurrent and adjuvant IO are being examined. Figure 5.2 demonstrates the variability in the length of adjuvant treatment. This is a collective strength of these trials and will improve our knowledge in this space.

5.2.4.2 Chemotherapy regimens

The variability of concurrent chemotherapy between trials and, in some instances, within trials may impact IO efficacy. Evidence from other tumour sites suggests a negative correlation between lymphopenia and IO efficacy[261,262]. Some trials allowed a choice between MCC or CisP. In the ACT2 trial, higher haematological toxicity was seen with MMC versus CisP, which impacted white blood cells specifically[67]. The choice of CisP or MMC may affect outcomes in these trials, given the possible synergistic relationship between CisP and IO[263]. Higher haematological toxicities are also likely in trials that used two doses of MMC or CisP instead of one, with some trials allowing either option. Given the equivalence of concurrent 5-FU/MMC and CAP/MCC, it is understandable why many trials allowed for a choice of fluoropyrimidine. However, when integrated with IO, this may be important, given the higher haematological toxicities from 5-FU[264]. In the future, RT-IO trials should aim for tighter chemotherapy protocols or consider appropriate statistical design where chemotherapy differences are likely to impact the primary trial outcome.

5.2.4.3 Radiotherapy regimens

Differences across these trials in RT regimens demonstrate the diversity in routine clinical practice worldwide. Differences in both elective RT volume and dose could impact IO efficacy. As performed in INTERACT-ION, the reduction in irradiated field volumes has preclinical and human data to support further investigation[133,265]. Given the impact on immune cell tumour infiltration, the inclusion of tumour-draining lymph nodes for RT in HNSCC may hamper RT-IO synergy and contribute to the negative results seen in these trials[266]. There are differences in elective pelvic volumes across ASCC RT contouring guidelines[267]. RT-IO trial protocols should include which contouring guideline was followed. Detailed RT information, including dose and volumes, should be included if a specific guideline was not followed. At least three trials in this review included RTQA. It is recognised that there is a lack of RTQA in Phase III clinical trials [268]. When feasible, it should be included in future RT-IO ASCC trials.

Regardless of nodal irradiation, a comparatively high elective RT dose will cause worse systemic lymphopenia. It can be speculated that a lower RT dose to elective nodes from brachytherapy compared to standard external beam radiation played a role in the success of KEYNOTE A-18[129]. There is interest in altering RT planning for ASCC outside of RT-IO trials. The DACG-II trial used a bone-sparing algorithm for RT planning to reduce pelvic insufficiency fractures. This may further reduce treatment-associated lymphopenia and could be considered for future RT-IO trials[269].

5.2.4.4 Statistical design

As a result of the unavailability of complete protocols or published results, there was a notable shortage of information on the statistical design of many of these trials. Only two had sufficient statistical detail regarding the design and sample size. The two trials where a protocol or publication was available but with no statistical design referenced

were phase Ib/II trials, with a limited sample size in one. This lack of detail reflects the need for considered statistical design in the setting of phase I radiotherapy-drug combination trials to improve evidence generation and the need for consistent reporting in early-phase clinical trials[163,270]. Given the rarity of ASCC, the sequential safety evaluation in CORINTH is prudent, albeit without a formal statistical design. Safety issues have arisen in comparable RT-IO trials for other cancers in the pelvis, with grade 3 toxicity in the first dose cohort of the PLUMMB trial for bladder cancer that resulted in a reduced radiotherapy dose for subsequent cohorts[271]. The pharmaceutical industry's interest and investment in developing treatments for ASCC could be jeopardised if the initial safety results are unfavourable. This interest is particularly precarious compared to other more prevalent and potentially more profitable cancers.

None of the trials identified explored multiple therapies or used biomarker stratification. The considerable variability in the type and timing of IO could be efficiently incorporated using a master protocol[272]. In the RT-IO setting, a master protocol allows differing regimens with the same drugs or different drugs to be tested in direct comparison with each other. Master protocols have been successfully used in ASCC-RT trials - PLATO trial incorporates ACT3, 4, and 5, investigating different doses of RT stratified for risk of treatment failure based on TNM staging[273]. There are also examples of successful master protocols in novel RT-drug combination trials where multiple agents are available for investigation. CONCORDE is a platform trial for patients with NSCLC receiving radical RT[274]. It is designed to assess multiple novel DNA damage response inhibitors in combination with RT, with participants randomised to receive a novel agent in combination with RT or RT alone in an unblinded fashion. This trial has a sizeable translational sample collection scheme and RT quality assurance (RTQA) embedded, allowing direct comparison between multiple drugs and detailed safety data on the combined effects of RT and each drug. A similar design could be used in a Phase I ASCC master protocol to evaluate multiple agents, acknowledging that the trials identified here were run in different countries. International collaboration would be required to run this type of complex study.

5.2.4.5 Biomarkers

Although many trials investigated biomarkers as part of their translational work, only INTERACT-ION used a biomarker (cHPV-DNA) as part of its criteria to assign CRT treatment. It is unclear whether any trial had an “integrated” biomarker – used to evaluate specific hypotheses with a defined objective and a statistical analysis plan[275]. An integrated biomarker for specific IO treatment needs good analytical and clinical utility[276]. Although data suggest PD-L1 from diagnostic tissue is prognostic for CRT in ASCC, heterogeneity in evaluation methods and the positivity cut off hinder comparisons and reduce its clinical utility[29,33]. This is likely an issue for other translational work from these RT-IO trials[29]. Tumour-infiltrating lymphocytes (TILs) and cHPV-DNA are other biomarkers of interest in ASCC with similar heterogeneity in

evaluation methods[91,277]. Collating techniques and cut-offs from published data in ASCC could be used as a first step to reduce heterogeneity in future trials.

5.2.4.6 Trial Endpoints

Variation in outcomes and endpoints will hinder our ability to compare these trials and has been previously addressed in CRT trials for ASCC. CORMAC was an international collaboration using the Core Outcome Measures in Effectiveness Trials method that resulted in 19 outcomes across four domains: disease activity, survival, toxicity, and life impact and should be considered by investigators for future ASCC trials[172]. These outcomes are a minimum rather than a prescriptive outcome set, and the use of CORMAC does not constrain the use of outcomes specific to IO trials, such as immune-related patient-reported outcomes or iRECIST. Although more relevant for phase II and III trials, CORMAC would make comparing results easier.

5.2.4.7 Patient selection

Variations in the definition of locally advanced disease could limit comparison between these trials in the future. This is further complicated by a recent update in AJCC/UICC TNM staging. Version 9 has updated definitions for Stage IIB and IIIA disease due to poorer 5-year survival for IIB disease (T1/2N1M0) compared to IIIA disease (T3N0/N1M0) in version 8[278]. Pooling data from RT-IO ASCC trials into an individual patient database could help make comparisons between trials and take account of differences in patient selection. The ARCAD Advanced Colorectal Cancer Database demonstrates the benefits of pooling patient data from international centres to optimise clinical trial data. ARCAD has resulted in 18 peer-reviewed papers and 28 published abstracts, with up to 18399 patients included in recent publications[279]. Alphanumeric TNM data and chemotherapy regimens are recorded clinically and are often part of data collection forms for clinical trials. Using a pooled database the unintended heterogeneity in patient selection, chemotherapy, and radiotherapy regimens found in this review could be leveraged to ascertain their impact on IO efficacy.

5.2.4.8 Translational analysis

Although helpful in generating hypotheses, RT-IO preclinical models have a range of challenges that mean they do not represent good surrogacy for in vivo human outcomes. It is recognised that RT-IO preclinical models should be as relevant as possible for the intended clinical setting[280]. Although preclinical models of ASCC are beginning to be developed, none have been evaluated using RT-IO combinations. Given the lack of RT-IO preclinical models specific to ASCC, translational analysis from these clinical trials is essential. Where data availability allowed, there was a considerable variation in the type of analysis planned and the timepoints at which these samples were collected. A “minimal translational component” for all trials, like the COS proposed by CORMAC, could be used in future trials. Globally, most patients

undergoing CRT for ASCC have weekly blood samples during treatment and have appointments 3 months and 6 months post-treatment. Translational timepoints should correlate with standard clinical visits and when decisions on treatment need to be made, such as at the end of CRT or recurrence. As with CORMAC, this is a minimum rather than a comprehensive recommendation. Additional components relevant to the specific IO in a trial may need to be included. Within translational blood samples, various processing options are available, ranging from cost-effective and easily storable, such as plasma for cHPV-DNA, to more costly ones requiring specific laboratory facilities, such as PBMCs for immunophenotyping. Both the number of timepoints and type of processing in this recommendation should not so prohibitive as to hinder the development of new trials. Figure 5.3 suggests a possible "minimal translational component" for RT-IO ASCC trials, recognising that a structured process of reaching consensus involving ASCC researchers internationally is required to develop this idea further. This could also be applied to future RT-drug combinations outside of IO, such as hypoxic-modifying agents. Many sample biobanks exist in different countries, but knowledge of what has been collected and when is not widespread. Collating this data could be used as a first step to develop this further.

5.2.4.9 Strengths and limitations

While systematic reviews on efficacy for RT-IO trials for other tumour types have been published, to our knowledge, this is the first systematic review evaluating different aspects of trial design for RT-IO combination trials[281]. The heterogeneity found in this review likely applies to RT-IO trials in other tumour types, and suggestions to reduce heterogeneity and improve comparisons in ASCC may be broadly relevant to many tumour types. However, there are several limitations to this study. The lack of protocol publication and full trial protocol for all trials hindered our ability to compare them, particularly for translational analysis. At least three protocols were not available due to commercial sensitivities. Data sharing for ASCC is more important than in more common cancers, with limited preclinical models and an inherently small number of possible trials investigating new treatment options. The poor functionality of the search interface on clinicaltrials.gov and EudraCT has been previously recognised [282]. The minimum level of detail required by clinicaltrials.gov and other trial databases is insufficient to compare trials adequately. When contacted, investigators for the RT-IO ASCC trials were happy to share more information, demonstrating that it would be feasible for trial databases to require a higher level of granularity for trial registration. Despite searching PubMed and performing an enhanced grey literature search, it is possible that some trials meeting the inclusion criteria were missed. Including the BISQUIT trial, which investigated the addition of probiotics and prebiotics to standard of care, CRT could be questioned as an IO. However, the proposed mechanism of action for this intervention was the modulation of the immune system[283].

The lack of formal trial comparison tools could be a weakness in this systematic review. Radiotherapy is a complex intervention with specific methodological considerations for trial design, and when combining this with different forms of IO, the utility of these tools is limited[284]. Although no statistical analysis was performed, this review was interested in clinical and methodological rather than statistical heterogeneity. Objective methods of assessing clinical and methodological heterogeneity are an area of unmet need[285]. With 14 patients across all trials having patient outcomes and no controls to compare these outcomes to, the lack of a meta-analysis is justified.

5.2.4.10 Recommendations

Across tumour types, trials combining IO with RT have been empirically rather than scientifically informed. It is important to explore various IO drugs and schedules with RT to find the optimal combination of RT-IO for patients with ASCC. The current trials investigating RT-IO combinations for ASCC do address this. Still, unintentional differences in chemotherapy, RT, and patient selection within and across these trials will make it difficult to compare their results. While some level of heterogeneity between these trials is unavoidable due to differences in national guidelines and centre protocols, there are several areas where we could improve our ability to compare, such as endpoint selection and translational analysis. Table 5.5 lists the key recommendations from this review.

Table 5.5 Summary of recommendations to reduce heterogeneity and improve comparisons for RT-IO ASCC trials

- Fully documented statistical design across all trial phases
- Use of master protocols to evaluate multiple IO regimens.
- Tighter chemotherapy trial protocols, with appropriate statistical design to account for differences that may impact the primary trial outcome.
- Use of high-quality RTQA where feasible
- Details of which radiotherapy contouring guideline has been follow in the trial protocol. If no guidelines are followed, details of radiotherapy dose and volumes should be included.
- Collate data on methods and techniques currently used for biomarkers of interest to achieve greater consistency across future trials.
- A “minimal translational component” to coincide with common clinical visits and when decisions on treatment need to be made, such as at the end of CRT or recurrence
- Individual patient data including TNM staging, radiotherapy regimen and chemotherapy regimen recorded within trials and shared on an individual patient-level database
- Consistent outcome selection and definition such as those recommended by CORMAC

- Trial protocols published or available on request, with more detailed minimum requirements for registration on trial databases such as clinicatrials.gov

5.2.4.11 Acknowledgements

Dr Lakshmi Rajdev and Professor Stefano Kim for providing translational details for NCI-EA2165 and SPARTANA trials, respectively

5.3 CORMAC-2

5.3.1 Introduction

The majority of ASCC patients present with localised disease where the primary treatment is CRT. Six published phase III randomised controlled trials provide much of the evidence supporting this approach[62–67].However, each trial reported different primary outcomes, and even when the same outcomes appeared to be used, definitions of these outcomes varied (Table 1.2). This limits between-trial comparison and has resulted in different chemotherapy and radiotherapy regimens being recommended internationally[286–289].

The Core Outcome Research Measures in Anal Cancer (CORMAC) project was an internationally ratified COS for trials of CRT for ASCC, developed through a consensus study involving 149 patient and healthcare professional participants from 11 different countries. The output from CORMAC-COS included 19 outcomes across 4 domains of disease activity, survival, toxicity, and life impact (Table 1.3) [172]

Whilst utilisation of the CORMAC-COS will go some way to harmonising outcome reporting in ASCC trials, standardised definitions for each of the outcomes in the COS are required to ensure quality and consistency in measurement and reporting. While COS have been developed for many disease areas, to date very few COS projects have followed through to the necessary next step of recommending standardised outcome definitions. Here the second phase of the CORMAC project (CORMAC-2) is presented, in which international consensus on standardised definitions of the 11 disease activity and survival outcomes in the CORMAC-COS was established.

5.3.2 Methods

Section 2.4 in the methods chapter details the patient and public involvement, selection of outcomes, systematic review update, Delphi questionnaire, consensus meeting, registration, and ethics.

5.3.3 Results

5.3.3.1 Search strategy and selection criteria

The search from the original CORMAC was updated using exactly the same search strategy, databases, and selection criteria. Full details have been published previously [171]. The updated systematic review identified 1646 outcomes from 190 trials and observational studies of CRT for ASCC. Outcomes and any accompanying definitions were extracted verbatim from included studies.

5.3.3.2 Systematic review & Delphi questionnaire design

Outcome definitions were extracted verbatim and then summarised to allow the identification of similar themes and concepts. For example, within the outcome "treatment failure," identified concepts included anatomical definition, timing of assessment, modality of assessment and grading of response. For composite time-to-event outcomes, all events were extracted, including definitions of events where provided. The summarised extracted outcomes and concepts were presented to and discussed by the steering committee and the discussion was used to inform the design of the Delphi questionnaire items.

For questions about modality of assessment and grading criteria for assessment of treatment failure and treatment response, participants were asked to separately consider small, low-risk tumours (T1-2 ≤4cm N0 or Nx anal canal or T2 ≤4cm N0 or Nx anal margin) and large, high-risk tumours (T2 N1-3 or T3-4 N- any). This was because the steering committee felt it likely that different modalities may be preferred based on risk stratification. The criteria used to distinguish these two groups were based on the PLATO trial protocol but it was emphasised to participants that this was just one example of risk stratification and that other definitions may be in use or adopted in future.[290] The round 1 questionnaire contained 67 options under 15 stem questions covering components of the definition and assessment of 9 outcomes.

5.3.3.3 Delphi questionnaire results

50 participants from 13 countries and 7 different healthcare disciplines took part in both rounds of the Delphi questionnaire (Table 5.6). As a result of participant suggestions after round 1, HRA was added to the modality of assessment of treatment failure in round 2. Participants in round 1 also fed back that the wording and concepts describing the anatomical definition of local and regional failure were unclear, particularly relating to bony involvement. The steering committee agreed that this would therefore be discussed at the consensus meeting regardless of Round 2 results. After both rounds of the Delphi questionnaire, 36 items met the consensus-in criteria, 24 consensus-out, and 9 reached no consensus. Overall, 10 out of the 15 questions reached agreement on all components and 5 questions had components reaching no consensus (Table 5.7). The attrition between Round 1 and 2 was 34%.

Table 5.6 Round 2 participants by country and profession

Country	Number	Profession	Number
Australia	1	Clinical Oncologist	15
Canada	1	Colorectal Surgeon	12
Germany	1	Medical Oncologist	6
Italy	1	Radiation Oncologist	12
Netherlands	2	Radiographer	1
Norway	1	Radiologist	3
Poland	1	Radiophysicist	1
Portugal	1		
Spain	1		
Sweden	3		
United Kingdom (UK)	29		
United States of America (USA)	7		
Uruguay	1		

Table 5.7 Round 1 questionnaire with Results after both round of Delphi questionnaire and consensus meeting. Green = Consensus In, Yellow = No Consensus, Red = Consensus Out

Domain	Outcome	Question	Option	Decision after Delphi questionnaire	Decision after consensus meeting
Disease Activity	Treatment response	Timepoint	2 months		
			3 months		
			4 months		
			6 months		
		Modality of assessment – small, low risk tumours	Clinical Examination (patient awake)		
			EUA		
			Biopsy		
			MRI SCAN		
			CT scan		
			PET-CT scan		
		Modality of assessment - Large, high risk tumours	Clinical Examination		
			EUA		
			Biopsy		
			MRI SCAN		

Domain	Outcome	Question	Option	Decision after Delphi questionnaire	Decision after consensus meeting
			CT scan		
			PET-CT scan		
		Criteria Grading	RECIST for imaging follow-up (for MRI or CT imaging)		
			Tumour Regression Grading (TRG) system for MRI imaging		
			Clinical RECIST from ECOG DECREASE		
			Metabolic response for PET-CT imaging		
			Clinical examination and imaging combination assessment: Complete response, partial response, residual thickening, no response		
	Treatment failure	Timepoint	2 months		
			3 months		
			4 months		
			6 months		
		Local failure	Primary tumour site within the anorectum		
			Primary tumour site including any directly invaded structures e.g. the vagina		

Domain	Outcome	Question	Option	Decision after Delphi questionnaire	Decision after consensus meeting
		Regional failure	Inguinal lymph nodes		
			Mesorectal lymph nodes		
			Presacral lymph nodes		
			Internal iliac lymph nodes		
			External iliac lymph nodes		
			Common iliac lymph nodes		
			Para-aortic lymph nodes		
			Any disease within the pelvis up to the level of the sacral promontory including the bones of the pelvis		
			Any disease within the pelvis up to the level of the sacral promontory excluding the bones of the pelvis		
		Distant failure	Any tumour deposits outside the pelvis		
			Any tumour deposits within the pelvis, including the bones of the pelvis, that are not nodal or primary tumour site		
		Radiation field	How important is it that the definition of local or regional failure includes information on whether the		

Domain	Outcome	Question	Option	Decision after Delphi questionnaire	Decision after consensus meeting
			site of failure is in the radiation planning treatment volume field		
		Modality of assessment – small, low risk tumours	Clinical Examination (patient awake)		
			EUA		
			Biopsy		
			MRI scan		
			CT scan		
			PET-CT scan		
			High resolution anoscopy (Additional Round 2 option)	asked	
		Modality of assessment - Large, high risk tumours	Clinical Examination (patient awake)		
			EUA		
			Biopsy		
			MRI scan		
			CT scan		
			PET-CT scan		
			High resolution anoscopy (Additional Round 2 option)	Not asked	

Domain	Outcome	Question	Option	Decision after Delphi questionnaire	Decision after consensus meeting
	Salvage Surgery		Abdomino-perineal resection		
			Pelvic exenteration		
			Wide local excision of tumour (without excision of the anorectum)		
			Excision of lymph nodes		
Survival	Anal-cancer specific survival		Death due to anal cancer		
			Death due to any cause when anal cancer is present		
			Death due to treatment for anal cancer		
	Disease-free survival		Local failure		
			Regional failure		
			Distant failure		
			New primary anal cancer		
			New primary HPV-related cancer		
			New primary cancer of any type		
			Death from any cause		
	Metastasis-free survival		Distant failure		

Domain	Outcome	Question	Option	Decision after Delphi questionnaire	Decision after consensus meeting
			Death from any cause		

5.3.3.4 Consensus meeting

12 participants from 4 different healthcare disciplines and 7 countries attended the online consensus meeting to ratify the options that had reached consensus-in or consensus-out through the Delphi questionnaire, and to discuss and vote on the options that had not reached consensus. All participants had completed both rounds of the Delphi questionnaire. Clarification of some of the options that had reached consensus-in or consensus-out after the Delphi questionnaire took place but no fundamental problems were raised, and all these items were ratified. Discussions regarding anatomy, bony involvement and radiation were structured using clinical examples to facilitate understanding and clarify definitions. It was accepted that the exemplar clinical scenarios used may be rare in clinical practice but necessary to ensure the utility and robustness of the final definitions. After completion of the Delphi questionnaire and consensus meeting, definitions were agreed for 7 outcomes comprising 16 aspects from 41 individually specified definitions (Table 5.8).

Table 5.8 CORMAC-2 Agreed definitions for disease activity and survival outcomes.
EUA: examination under anaesthesia. MR: magnetic resonance. CT: computer tomography. PET-CT: positron emission tomography

DOMAIN	OUTCOME	ASPECT	DEFINITION
Disease activity	Treatment response	When treatment response is measured	3 months 6 months
		Modalities used to assess treatment response - small, low risk tumours	Clinical examination MRI scan
		Modalities used to assess treatment response - large, high risk tumours	Clinical examination MRI scan
		Criteria used to assess treatment response	Clinical examination and imaging combination assessment: Complete response, partial response, residual thickening, no response. Tumour Regression Grading (TRG) system for MRI imaging
	Treatment failure	When treatment failure can be assessed	6 months
		Anatomical locations are included in local failure	Primary tumour site within the anorectum Primary tumour site including any directly invaded structures e.g. the vagina
		Anatomical locations are included in regional failure	Inguinal lymph nodes Mesorectal lymph nodes Presacral lymph nodes Internal iliac lymph nodes External iliac lymph nodes

DOMAIN	OUTCOME	ASPECT	DEFINITION
			Common iliac lymph nodes And/or any disease within the pelvis up to the level of the sacral promontory excluding the bones of the pelvis
		Anatomical locations are included in distant failure	Any tumour deposits outside the pelvis Any tumour deposits within the pelvis, including the bones of the pelvis, that are not nodal or primary tumour site
		Additional anatomical information on treatment failure	Definition of local or regional failure includes information on whether the site of failure is inside the radiation clinical target volume (CTV)
		Modalities used to assess treatment failure - small, low risk tumours	Clinical Examination Biopsy MRI scan
		Modalities used to assess treatment failure - large, high-risk tumours	Clinical Examination Biopsy MRI scan CT scan PET-CT scan
	Salvage Surgery	Procedures included in the definition of salvage surgery	Any procedure to excise recurrent/residual tumour following primary chemoradiotherapy. Including but not limited to abdomino-perineal resection; pelvic exenteration and lymphadenectomy
Survival	Overall survival	Events in overall survival	Death due to any cause
			Death due to anal cancer

DOMAIN	OUTCOME	ASPECT	DEFINITION
	Anal-cancer specific survival	Events in anal-cancer specific survival	Death due to any cause when anal cancer is present Death due to treatment for anal cancer
	Disease-free survival	Events in disease-free survival	Local failure Regional failure Distant failure Death due to any cause
	Metastasis-free survival	Event in metastasis-free survival	Distant failure Death due to any cause

5.3.4 Agreed outcome definitions

Each outcome definition is given below. Where relevant, the nuances from discussion at the consensus meeting are given to explain the decision-making fully and transparently.

5.3.4.1 Treatment response

The outcome “treatment response” is an assessment of the response of the primary tumour and involved lymph to treatment. In defining this outcome, three components were considered: The timepoint at which the assessment of response is made, the modality with which the assessment is made and how the response is graded.

5.3.4.1.1 Timepoint

Treatment response assessment should take place at 3 months and 6 months after completion of CRT

5.3.4.1.2 Modality of assessment (for small, low-risk tumours and large, high-risk tumours)

1. Clinical Examination (patient awake)
2. MRI scan

Both clinical examination with the patient awake and an MRI scan should be used to assess treatment response. It was agreed that a CT scan was not chosen for the modality of assessment (for both small, low-risk and large, high-risk tumours) on the

assumption that an MRI scan is performed. If an MRI scan is not available, a CT scan with contrast should be used instead.

5.3.4.1.3 Assessment criteria/grading

1. Clinical examination and imaging combination assessment – Categorised as complete response, partial response, residual thickening, no response.
2. Tumour Regression Grading (TRG) system for MRI imaging.

A combination of clinical examination and imaging should be used to classify response into complete response, partial response, residual thickening, or no response. The TRG system for MRI imaging should be used, which categorises response from Grade 1 (complete response with no evidence of tumour and normal appearance of the anus) to Grade 5 (no response of the primary tumour or frank tumour progression).[291] It was recognised that while TRG for MRI imaging is not widely used, mandating it in a trial setting is easier and important to ensure standardisation and reduce heterogeneity.

5.3.4.2 Treatment failure

The outcome "treatment failure" describes the presence of disease at a specified timepoint after completion of CRT and makes no distinction between persistent or recurrent disease. In defining this outcome, three components were considered: The timepoint at which treatment failure can be defined, the definitions of local, regional, distant and radiation field failure, and the modality by which treatment failure can be defined.

5.3.4.2.1 Timepoint

Treatment failure should be assessed at 6 months after completion of CRT

5.3.4.2.2 Anatomical definitions

Local failure is defined as disease at the primary tumour site within the anorectum including any directly invaded structures e.g. the vagina

Regional failure is defined as disease involving the inguinal, mesorectal, presacral, internal, external iliac, or common iliac lymph nodes. A soft tissue deposit below the sacral promontory that is not from the primary tumour or nodal is also considered a regional failure. Distant failure is defined as any tumour deposit outside the pelvis, or any bony lesions that do not arise from direct invasion by the primary tumour or a regional node.

It was agreed through discussion and voting at the consensus meeting that direct bony invasion from the primary tumour is a local failure, bony invasion from a node is a

regional failure and any bony invasion not arising from the primary tumour or node is a distant failure.

5.3.4.2.3 Radiation field

It was agreed that in addition to information about the anatomical location as above (local, regional, or distant), treatment failure should include information on whether the failure is within the radiotherapy clinical target volume (CTV).

5.3.4.2.4 Modality of assessment (for small, low-risk tumours)

1. Clinical Examination (patient awake)
2. Biopsy
3. MRI scan

At the consensus meeting it was clarified that in the context of an ASCC clinical trial, confirmation of treatment failure (for both small, low-risk and large, high-risk tumours) should involve histological evidence of invasive disease, whether this is from a biopsy or a surgical resection specimen. Although it is normal practice to have histological confirmation of treatment failure from a biopsy prior to salvage surgery, this is not mandatory in all situations, and confirmation can come from the surgical resection specimen.

5.3.4.2.5 Modality of assessment (for large, high-risk tumours)

1. Clinical Examination (patient awake)
2. Biopsy
3. MRI scan
4. CT scan
5. PET-CT scan

It was clarified that contrast enhanced CT imaging is required for the assessment of treatment failure in large, high-risk tumours. If PET-CT is performed and includes a contrast CT component, separate contrast enhanced CT is not required. After discussion, it was agreed that PET-CT for assessment of treatment failure in large, high-risk tumours was recommended but not mandatory. The integrity of a trial would not be impacted if PET-CT were not available.

5.3.4.3 Survival

5.3.4.3.1 Overall survival

Events in OS are death due to any cause. This was not asked in the Delphi questionnaire due to unanimous agreement in the literature and amongst the steering committee.

5.3.4.3.2 Anal-cancer specific survival

Events in anal-cancer specific survival are death due to anal cancer, death due to any cause when anal cancer is present and death due to treatment for anal cancer.

5.3.4.3.3 Disease-free survival

Events in DFS are local failure, regional failure, distant failure, and death due to any cause.

5.3.4.3.4 Metastasis-free survival

Events in metastasis free survival are distant failure and death due to any cause.

5.3.4.4 Salvage surgery

Discussion about what constituted salvage surgery concluded with unanimous agreement that salvage surgery is any surgical procedure to excise recurrent or residual tumour following primary CRT, including but not exclusive to abdomino-perineal resection, pelvic exenteration and excision of lymph nodes. It was also agreed that the details of the procedure undertaken as salvage surgery should be specifically reported in trials.

5.3.5 Discussion

CORMAC-2 builds on the CORMAC COS and provides the first internationally agreed definitions for outcomes relating to disease activity and survival for clinical trials of CRT for the treatment of ASCC. All included definitions were agreed by expert healthcare professionals from thirteen different countries and across the spectrum of disciplines involved in ASCC care and trials. The definitions were agreed using robust and transparent consensus methods to ensure equal representation from all participants. The aim of any COS is to encourage standardised reporting of outcomes in a particular health area. COS utilisation varies across health domains but even when used, outcome definition differences continue to reduce the capacity for data synthesis[292] .It is also increasingly recognised that understanding the breakdown of composite endpoints is necessary to interpret trial results accurately and gauge the true benefit of an intervention[293]. It is therefore recommended not only that all future trials evaluating CRT for ASCC use the CORMAC-COS, but also adopt the outcome definitions agreed in CORMAC-2.

The use of metabolic response assessment from PET-CT did not reach the threshold for inclusion after the Delphi questionnaire. Although not recommended by 2021 ESMO guidelines, PET-CT is increasingly used as part of response assessment in the UK and Europe, with data showing that combined PET-CT and MRI response assessment can predict subsequent outcomes better than either modality alone[289,294]. Outside of a

trial, in the USA and Canada PET-CT for treatment response assessment cannot be covered by insurance[34]. The variation in the availability of PET-CT in routine care may partially explain why it did not reach consensus in the Delphi questionnaire. Lack of standardised criteria for PET-CT response is also a recognised barrier to its implementation in routine clinical practice[294,295].

The definitions in CORMAC-2 were agreed upon for healthcare settings with no resource restraints. The "ideal" setting and trial recommendations should be to drive improvement in patient outcomes. For this reason, the TRG system for MRI reporting was included in the treatment response criteria definition despite its use not being currently universal. In settings where access to MRI and PET-CT is limited, CORMAC-2 is less relevant. Trials in these settings need pragmatic outcomes that enhance participation and real-world relevance within the limitations of available resources[296]. Some areas of the CORMAC-2 have cost-effective modifications in these settings, such as determining HPV status through IHC. Some components of definitions that did not reach threshold for inclusion following the Delphi questionnaire and consensus meeting may still be of interest in future trials. For example, secondary HPV cancer such as new diagnosis of HPV-associated head and neck cancer as an event for DFS was discussed at the consensus meeting. Although this was not included after voting, all participants felt that this is an important issue that is currently not well researched or reported and future trialists should consider including secondary HPV-associated cancer as an additional outcome beyond those in the CORMAC COS[36].

The distinction specified between large, high-risk, and small, low-risk tumours within the disease activity outcome definitions reflects current clinical practice and understanding of prognostic factors for ASCC. T-stage and N-stage are the most reliable clinical prognostic factors that stratify current clinical guideline recommendations[10]. TNM staging is used to stratify patients into different trials as part of the PLATO trial, with escalation or de-escalation of standard CRT doses explored within each cohort and the DECREASE trial is also investigating dose de-escalation for early stage disease[290,297].Risk stratification in future trials will likely evolve as the understanding of ASCC biology improves[298]. Future trial stratification may be based on biomarkers, such as HPV status or TILs[26]. In HPV+ disease, adaptive treatment based on cHPV-DNA monitoring is likely both for escalation and de-escalation trials.

PFS was included as a core outcome in the CORMAC-COS. At the time, questions in the literature about its validity as marker for improved survival or QOL were noted however as it met consensus criteria on voting it was included in the CORMAC-COS[171,299–302]. After gathering all existing definitions for PFS during the first phase of CORMAC-2, it became clear that the event "disease progression" was frequently included in definitions of PFS, however it was rarely further defined. Where disease

progression was defined it was frequently described as treatment failure, which rendered the definitions of DFS and PFS effectively the same. Following careful consideration and discussion by the steering committee, it was decided that PFS was of limited relevance outside the context of trials of palliative interventions or metastatic disease. The scope of the CORMAC-COS is trials of CRT for non-metastatic ASCC with curative intent and therefore the decision was taken not to include PFS in CORMAC-2.

Whilst the difficulty caused by unclear and inconsistent definition of outcomes in cancer trials have been widely reported, there remains very little published guidance or recommendations on standardised outcome definitions, and where is has been produced it has often been without formal consensus[171,302–305]. In response to this issue back in 2012, Bonnetain and colleagues planned a series of projects to define time-to-event (TTE) endpoints in cancer trials (DATECAN). Recommendations were published for TTE endpoints in breast, localised colon, renal, and pancreatic cancer, and for gastrointestinal stromal tumous[178,306–309]. No further work from the group has been proposed.

A systematic review undertaken by the COMET initiative in 2020 found that only one-third of COS published up until 2018 made any recommendation on how outcomes should be defined or measured[310]. The focus of this review however was on outcomes requiring a measurement instrument (e.g. patient reported outcomes such as physical function) rather than clinical or oncological outcomes requiring a definition. Furthermore, this review showed that even where instruments were recommended, many studies did not meet the recommended standards for identifying and selecting outcome measurement instruments.

The CORMAC project is one of very few COS projects registered on the COMET database that has gone on after establishing COS to complete the crucial next step of agreeing standardised outcome definitions and measurement recommendations. Whilst there are recommendations for best practice in how to identify and select outcome measurement instruments, as yet there is no recommended approach for agreeing outcome definitions[176]. The methods employed in CORMAC-2 were therefore developed based on the recommended consensus methods used for COS development and the methods proposed by the DATECAN initiative. A-priori publication of the CORMAC-2 protocol, clear and transparent reporting of the methods used in each stage, incorporation of patient and carer views and involvement of a broad pool of global experts minimised the potential for bias and maximised our confidence in the final agreed outcome definitions.

One limitation of the CORMAC project is the restriction to the English language which likely limited participation in countries where English is not widely spoken and

contributed to the larger proportion of participants from the UK and USA. Although many of the trials within the scope of the CORMAC COS are conducted in the UK and USA, there are ongoing trials in Germany and France[137,255]. Whilst there was representation from all disciplines of healthcare professionals involved in ASCC care and trials, there was a preponderance of clinical/radiation oncologists. Globally, most ASCC trials are led by clinical/radiation oncologists. Therefore, apart from low participation from Germany (one participant) and France (none), the participants in CORMAC-2 are arguably representative of the groups most likely to use the CORMAC-COS.

Four steering committee members are investigators in the PLATO trial, which could have influenced the content or wording of the Delphi questionnaire. However, the impact of this was minimised through invitation for alternative definitions from all Delphi participants in round 1. Given the differences in outcome definitions between the PLATO protocol and CORMAC-2, and dropping PFS from CORMAC-2 despite it being the planned secondary outcome for PLATO, this potential influence seems to have been minimal.

Although Delphi is a well-recognised and utilised consensus methodology in healthcare research, it does have some limitations. In Delphi methodology, participants do not engage in direct discussions until the consensus meeting. This contrasts with the nominal group methodology, where brainstorming occurs at the beginning of the process. It is possible that wider discussion of questionnaire design and wording beyond the steering group before the Delphi questionnaire would have improved the original questionnaire. However, during the first round of the Delphi, participants were given the opportunity to suggest new definitions that had not been included and to give free-text feedback, to ensure maximal inclusion of all potential options before proceeding to subsequent rounds. Additionally, the CORMAC-2 consensus meeting was chaired by experienced facilitators ensuring open discussion and lively debate that explored the nuance of practical application of the outcome definitions and significantly improved clarity on the “no consensus” prior to repeat voting.”

The number of participants at the consensus meeting was relatively small compared to the number of participants in the Delphi questionnaire. Consensus meeting participants had to have completed both rounds of the Delphi questionnaire to ensure that all participants were fully informed and engaged with the complex topics to be discussed. The number of consensus meeting participants was carefully considered in an effort to balance ensuring meaningful engagement and discussion between participants with adequate and balanced representation from a spectrum of healthcare professionals. Care was taken not to allow a small number of consensus meeting participants to overturn the results of a larger consensus from the Delphi. Only

outcome definitions had not reached consensus through the Delphi or in need of further clarification were discussed and voted on at the consensus meeting.

CORMAC has been cited in updates and studies in the field of ASCC but has yet to be cited in new trial protocols[10,311–314] CORMAC-2 addresses the lack of accompanying definitions and outcome measurement recommendations for disease activity and survival, but recommendations for measurement instruments for toxicity and life-impact outcomes are still pending. This is an area of active research in ASCC. Since the original CORMAC-COS was published, the QLQ-ANL27 health-related quality of life questionnaire for ASCC has now completed final international validation[315,316] . The ANCHOR trial, which found that the risk of ASCC was reduced with treatment for high-grade squamous intraepithelial lesions compared to active monitoring in patients living with HIV, developed a validated health-related symptom index which may have relevance to patients undergoing CRT for ASCC[317,318]. The next phase of CORMAC will be to complete an evaluation of available instruments and recommend specific measurement instruments.

Utilisation of the CORMAC-COS and CORMAC-2 standardised definitions in future trials could significantly improve the quality and utility of data available to inform clinical care. Incorporating them in PLATO and ECOG-DECREASE clinical trials is planned and will further promote awareness and uptake. Data sharing projects such as atomCAT, which uses distributed learning to compare factors associated with outcomes in ASCC across international centres, will greatly benefit from standardised outcome definitions[319].

In conclusion, by agreeing on definitions for outcomes in the domains of disease activity and survival, CORMAC-2 will facilitate greater use of the CORMAC COS, increasing outcome standardisation across trials, increasing the quality of data available for clinical decision-making, and ultimately enhancing patient care.

5.4 Relevance of CORMAC-2 to a future RT-IO ASCC Trial

CORMAC-2 deals with trials endpoint heterogeneity in ASCC and would typically be used in Phase II/III trials, rather than early-phase biologically driven trials, which are the main focus of this PhD. However, the techniques, ability, and experience of CORMAC-2 can be used to reduce heterogeneity in any stage of ASCC research. Reducing heterogeneity in earlier stages of research, prior to developing a clinical trial will improve the overall efficiency of translational research. In this thesis, heterogeneity has been identified at all stages of bench-to-bedside research.

The most apparent but unavoidable variability is the difference in biology between species and the genetics inherent in preclinical models, as well as their biological

relevance to a diverse human patient population. Heterogeneity in dosing and the schedule of both RT and IO in preclinical models raises concerns about how adequately they represent human RT-IO regimens. There is methodological heterogeneity in classifying Tregs and in how cHPV-DNA is performed and analysed. Heterogeneity in translational analysis arising from sample type, timing, and the laboratory techniques used limits the usefulness of reverse translation. All of this variability makes the transition from biological insights to improving standard of care much harder.

Delphi methods are currently used to get consensus on clinical trial endpoints, but the principles could be applied to earlier stages of clinical research to help improve efficiency. Open data repositories, distributed learning, and digital pathology are three technologies that could be leveraged to enhance comparisons and speed up translational research from bench to bedside.

Chapter 6 Conclusion

6.1.1 Key findings aim 1

"To identify pre-CRT and CRT-induced cellular markers of ASCC immune resistance and to correlate these with 6-month treatment failure (circa 25-30%) and complete response (circa 70-75%)."

Using flow cytometry for immunophenotyping of PBMCs, immune resistance markers related to peripheral Treg population, TIGIT expression and CTLA-4 expression were found to be associated with 6-month treatment failure in patients with locally advanced ASCC receiving CRT.

The peripheral Treg population, defined as FOXP3+CD4+CD25^{high}CD127^{low}, was statistically higher at baseline in patients with treatment failure and remained so during and after CRT. In both treatment failure and complete response groups, this population marginally increased during CRT and dropped back to levels similar to baseline by 6 months.

CTLA-4 expression was statistically higher on Tregs, CD8+ T-cells, and CD4+ T-cells at baseline in patients with treatment failure at 6 months. CTLA-4 dynamics differed between cell types. CTLA-4 expression on Tregs increased during and after treatment in patients with treatment failure but remained relatively stable for patients with complete response. In both CD4+ and CD8+ T-cells, CTLA-4 expression did not change in response to CRT until 6 months after treatment, where CTLA-4 expression in the treatment failure group reduced to a similar level as complete response.

TIGIT expression was statistically higher on CD4+ T-cells, with a trend for higher expression on Tregs, CD8+ T-cells and NK cells. CD112 and CD155 are the most specific ligands for TIGIT. CD112 was statistically higher on monocytes, with a trend for higher expression of CD155 and CD112 on B-cells. Across all cell types, there was little change in TIGIT expression in response to CRT. This includes NK cells, which showed faster and larger increases in expression of all other immune markers measured.

Across all cell types, CTLA-4 and TIGIT expression was highest on Tregs. Given the above results, targeting peripheral Tregs, CTLA-4 and TIGIT are three distinct but related immune resistance markers that may improve outcomes for patients with locally advanced ASCC. Given the dynamics of Tregs, CTLA-4 and TIGIT expression in response to treatment, a neoadjuvant approach is the most rational for all three markers. This is particularly so for Tregs and TIGIT, which are higher at baseline and showed limited response to CRT, suggesting a pre-CRT immune resistance mechanism. For CTLA-4, the dynamics on Tregs suggest a concurrent approach may also be

appropriate, given the marked increase in expression during CRT in patients with treatment failure compared to complete response.

6.1.2 Key findings Aim 2

"To identify pre-CRT and CRT-induced soluble markers of ASCC immune resistance and to correlate these with 6-month treatment failure (circa 25-30%) and complete response (circa 70-75%)."

A Multiplex human screening panel and TGF-β panel found no significant differences in cytokines measured in peripheral plasma between patients with complete response or treatment failure. Although measurement of IL-2Rα and LIF in plasma in future RT-IO ASCC trials is warranted as part of the exploratory translational analysis, large screening panels using Multiplex technology should not be a focus for future work RT-IO ASCC trials.

6.1.3 Key findings Aim 3

"To rationally inform and assist novel early-phase clinical trial design to integrate IO into future curative ASCC trials. Using biological outputs from Aims 1 and 2, a novel RT-IO ASCC trial design will be proposed."

As part of rationally developing future curative ASCC trials, a systematic review of all current ASCC RT-IO trials was performed, and the second stage of the CORMAC-COS was completed. The systematic review found that most RT-IO trials in ASCC were investigating PD/PD-L1 checkpoint inhibitors, with a TIGIT inhibitor, a cancer vaccine, and modulation of the immune system with pro/prebiotics also being investigated. All variations of neoadjuvant, concurrent and adjuvant IO regimens were used in these trials. Whilst they will give some information on the optimal timing of IO with CRT for ASCC, unintentional differences in chemotherapy, RT, and patient selection within and across these trials will make it difficult to compare their results. While some level of heterogeneity between these trials is unavoidable due to differences in national guidelines and centre protocols, there are several areas where we could improve our ability to compare, such as endpoint selection and translational analysis.

Heterogeneity in endpoint selection was addressed in CORMAC-2, which developed the first internationally agreed definitions for outcomes related to disease activity and survival for clinical trials of CRT for the treatment of ASCC. The techniques, ability, and experience to reduce heterogeneity in endpoint selection could be applied to other areas relevant to RT-IO ASCC trials, such as translational analysis or agreed reporting and classification of Tregs.

6.2 Future work

Whilst the findings from this thesis show significant promise, further work including validation of these results are required to inform the design of a biomarker driven, adaptive clinical trial. As per the CRUK prognostic/predictive biomarker roadmap, we must ensure they are accurate and reproducible in the target population. A first step in this could be to formally compare the peripheral Treg classification and association with treatment outcome from this thesis to that in the German publication discussed in 3.19.1 [184]. PBMCs have been collected in comparable Norwegian and Danish cohorts, and prospective analysis on these retrospective cohorts could help validate all three biomarkers. In each cohort, refinement of a cut-off for a "High peripheral Tregs" population could be assessed, which could be used as an inclusion criterion in a future trial. Given that the majority of patients with ASCC have a complete response to CRT, a more targeted trial population is warranted.

There are many more biobanks/sample collections that have diagnostic FFPE tissue rather than PBMCs. If planned multiplex IHC finds a good surrogate marker of peripheral Tregs in tissue, this could be more widely prospectively analysed, including in the ACT5 cohort. Standard operating procedures and assay performance assessment are also required before a clinical trial could start recruitment. The work-up and design of a biomarker focused, adaptive clinical trial requires significant specialised biostatistical input, and detailed discussion and development as part of a multidisciplinary team. The design of a future RT-IO trial will be performed as part of the PLATO2 consortium of which I and the work in this thesis will contribute towards that provides an excellent opportunity to combine the scientific findings with the necessary methodological and clinical expertise

In Chapter 3, low-dose metronomic cyclophosphamide, botensilimab, and tiragolumab were proposed as therapeutics that could target peripheral Tregs, CTLA-4, and TIGIT, respectively, in future ASCC trials. Much more research is needed to justify their use in this setting. Assessment of these markers in the ASCC cell lines and from the patient tissue collected as part of GRECIAN is currently underway. If results are promising, modulation of these markers in response to these agents in the ASCC-specific preclinical models described in 1.1.14 could then be performed. Results from the BICCC trial described in Chapter 3 and positive RT-IO trials in other tumour sites would also support the use of these drugs in ASCC.

Further work in clinical trial methodology could improve the design of a future ASCC RT-IO trial. As discussed in Chapter 5, a "core translational component" would improve comparisons between trials and help develop our understanding of ASCC biology. The next stage of CORMAC, to agree on the instruments and definitions in the toxicity and

life impact outcome domains, would further increase trial standardisation and improve comparisons.

Rapid advances in cHPV-DNA provide an exciting opportunity to adapt novel interventions in a timely manner in future ASCC trials. As discussed in Chapter 4, it was intended that cHPV-DNA measurement using the HPV-detect assay developed by Bhide and colleagues at the ICR would be performed on the GRECIAN cohort. This will be performed in early 2025. Figure 6.1 to Figure 6.3 show the results from Bhide and colleagues, and Spindler and colleagues that we hope GRECIAN will replicate. These show that cHPV-DNA can be a good marker for response to CRT, and that negative/below threshold measurement at 12 weeks after CRT correlates with complete response. Figure 6.3 shows that in patients with negative cHPV-DNA at end of treatment, 87.5 % (28/32) patients went on to have a complete response. cHPV-DNA could form part of an adaptive design in future ASCC trials.

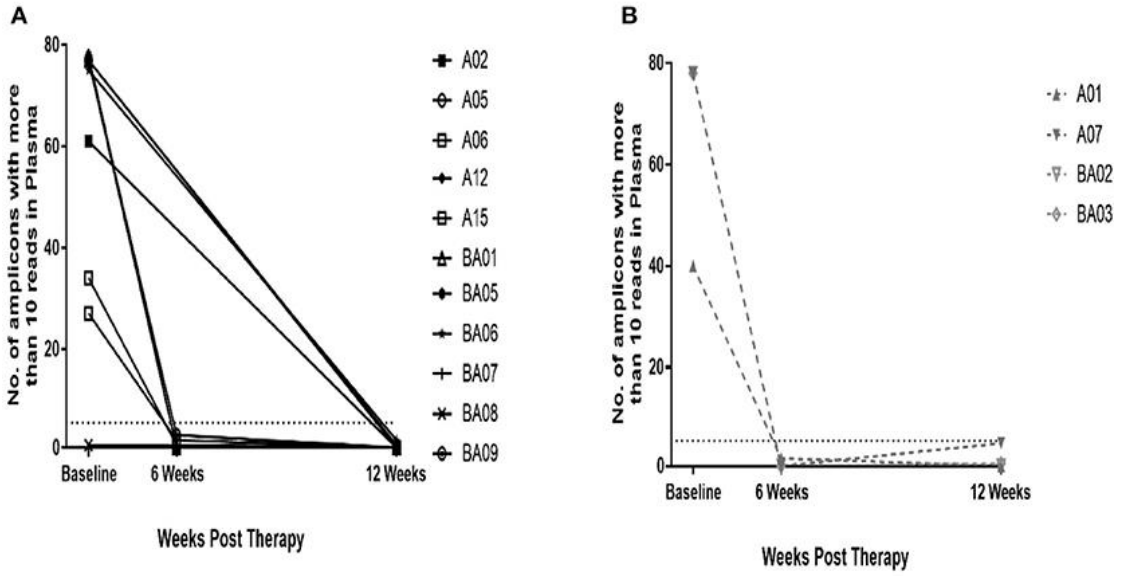


Figure 6.1 A shows 11 patients had complete response that had cHPV-DNA below threshold. B shows 4 patients with partial response 12 weeks after treatment, with complete response at 6 months. Taken from Bhide et al. 2020

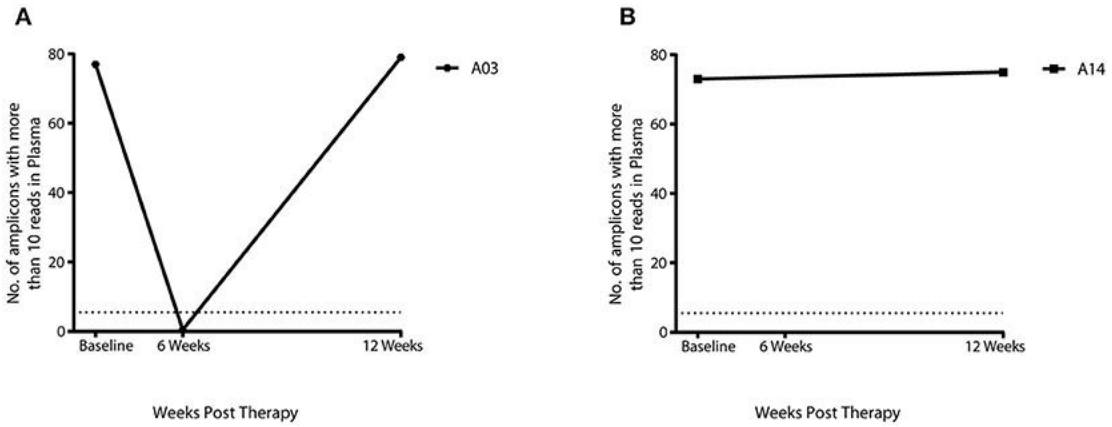


Figure 6.2 A shows 1 patient with residual disease at 12 weeks, B shows 1 patient with complete local response on DRE/MRI with distal relapse at 9 months. Taken from Bhide et al. 2020

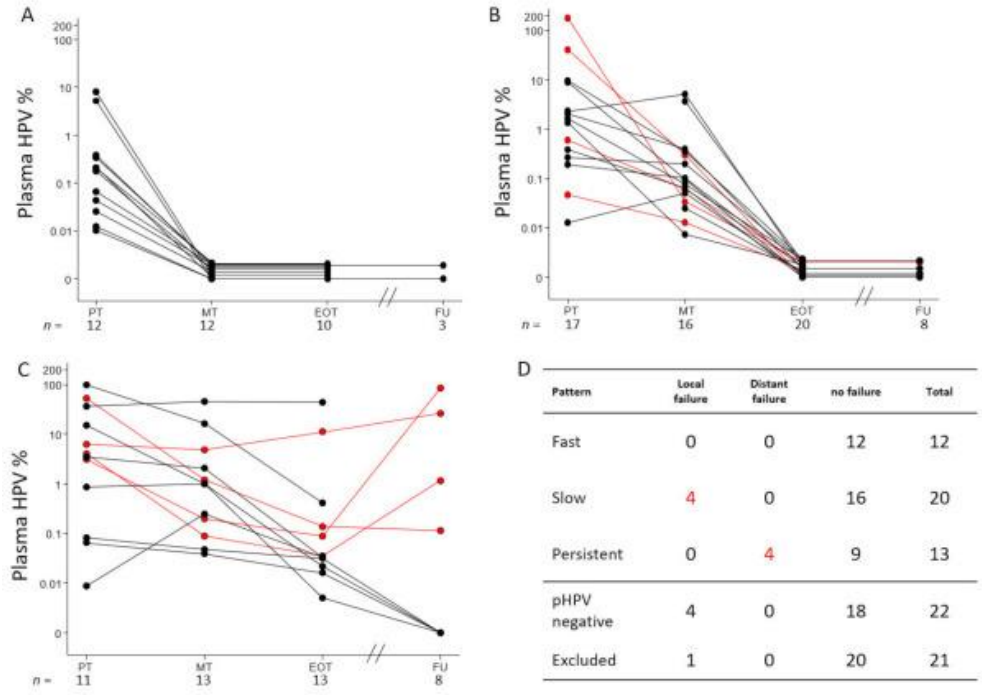


Figure 6.3 Elimination patterns of pHPV. Patients with measurable pHPV during treatment were divided into three groups. (A) 12 patients with a fast pHPV elimination pattern. (B) 20 patients with slow pHPV elimination pattern. (C) 13 patients with molecular persistent disease. In (D), the relation between the pattern and treatment outcome are outlined, including 22 patients with undetectable pHPV and the 21 patients who were excluded due to insufficient repeated pHPV measurements. Taken from Spindler et al. 2021

6.3 Limitations

This thesis has several limitations. First, the relatively small sample size restricts the conclusions that can be drawn, especially given that treatment failure is a rare event.

While recruitment could have been extended to additional centres, coordinating sample collection and ensuring consistent sample processing was already challenging with three centres involved. Manchester, Leeds, and Oxford are the top recruiters for PLATO, and the effort of expanding recruitment to other centres would likely have had diminishing returns. Extending the recruitment period was another option, but this would have compromised the timeline required to complete the analysis within the scope of a PhD thesis.

Additionally, performing multiplex IHC and RNA sequencing on diagnostic tissue earlier in the thesis would have provided stronger supporting data for the results from PBMCs. Similarly, incorporating cHPV-DNA analysis early would have been valuable, as comparing HPV-DNA clearance to cellular immune responses could yield important insights. A more comprehensive flow cytometry panel, particularly for classifying Treg populations in greater detail, would have enhanced the results. However, these limitations are insights gained in hindsight, and a PhD program is as much a learning programme as it is an opportunity for research production.

6.4 Study impact

The laboratory results described in this thesis are the first to describe the dynamic changes in immune response to CRT in patients with ASCC, identifying three different markers of immune resistance. These findings significantly contribute to the small yet growing body of research in translational science related to ASCC, enhancing our understanding of ASCC biology. To my knowledge, the systematic review of ASCC RT-IO trials is the first attempt to assess the impact of trial design on the comparison of trial results, and the recommendations made are broadly applicable to RT-IO trials across other tumour types. CORMAC-2 stands out as one of the very few examples of a registered COS project aimed at agreeing on definitions for outcomes and exemplifies the methodology required to reduce trial heterogeneity. Given its dual focus on biology and trial methodology, this thesis uniquely bridges the divide between laboratory and clinical research, aiming to improve clinical care for patients with ASCC. The laboratory results, the RT-IO systematic review, and CORMAC-2 will all contribute to rationally developing the next generation of personalised drug-chemoradiotherapy combination trials in anal cancer.

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Appendix A – PROSPERO for Systematic Review

Systematic review of radiotherapy-immunotherapy combination trials in squamous cell anal cancer

Citation

Robert Samuel, Adel Samson, Sarah Brown, David Sebag-Montefiore, Andrew Renehan, Natalie Cook. Systematic review of radiotherapy-immunotherapy combination trials in squamous cell anal cancer. PROSPERO 2023 CRD42023384068 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42023384068

Review question

What are the trial designs, treatment regimes and translational endpoints for radiotherapy-immunotherapy combination trials in squamous cell anal cancer?

Searches [1 change]

Sources:

PubMed (filtered for "Trials")

ClinicalTrials.gov

EudraCT

Additional search strategy information can be found in the attached PDF document (link provided below).

No time limits on publication. Only English language.

Types of study to be included

Inclusion criteria

• Interventional clinical trial, Phase I to IV inclusive.

• Trials including other tumour types where separate analysis of >5 anal cancer patients is possible

Exclusion criteria

• Non-interventional trials.

• Study protocol registered but study withdrawn or never occurred.

Condition or domain being studied

Squamous cell carcinoma of the anus.

Participants/population

Inclusion criteria

• Squamous cell carcinoma of the anus or anal canal, metastatic or locally advanced.

Intervention(s), exposure(s)

Inclusion criteria

• Any intervention combining radiotherapy with treatments to modulate or stimulate the immune system.

Exclusion criteria

• Studies investigating the immunomodulatory effects of current standard of care treatment, where the principle recognised mechanism of action of treatment is not immunomodulatory.

Comparator(s)/control

No comparators or controls.

Main outcome(s) [1 change]

1) Trial design - What phase of trial is being performed? Is it randomised? Are any novel designs being used? Is it stratified with biomarkers?

2) Treatment regime used - how is radiotherapy and immunotherapy being combined and how is this combination justified?

3) Translational endpoints - what samples are taken, at what timepoints are they taken and what analysis is being performed

Primary outcome is trial design. This review aims to assess the heterogeneity in clinical trial design. The clinical relevance is that heterogeneity across trials will hinder pooled analysis and not allow us to know which treatment is best for anal cancer patients.

Additional outcome(s)

None.

Data extraction (selection and coding)

Data will be recorded in a data collection form on microsoft word. If no trial protocol is available, authors will be contacted.

Data to be extracted

NCT number

Protocol available?

Anal cancer only? If not, what others are included

Stage of disease

Experimental arm

Drug regime

What does the dosing regime appear based on?

Primary Endpoints

Secondary Endpoints

Patient eligibility

Radiotherapy regime

Imaging regime

Geographical location

Phase of trial

Number of experimental arms

Randomised?

Biomarker selection

(Proposed) sample size

Finished, recruiting or setting up?

Translational/exploraotry analysis

Trial Design.

Risk of bias (quality) assessment

Cochrane Risk of Bias tools will be used depending on the trial design - RoB 2 and ROBINS-I for example.

Strategy for data synthesis [1 change]

Meta-analysis is not planned. It is expected that there will be large variation in trial design, treatment regime and translational endpoints, hindering formal data synthesis. Visual demonstration of variation through graphs and tables for these outcomes is planned.

As results of these trials are likely not to be available, a formal cochran Q's will not be possible. Therefore a formal narrative analysis will be performed. This will follow the Synthesis Without Meta-analysis (SWiM) reporting guideline. The author's of this systematic review are heavily involved in anal cancer trial design and will be able to provide context to these results.

Analysis of subgroups or subsets

No planned investigation of subgroups.

Contact details for further information

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Organisational affiliation of the review

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Type and method of review

Intervention, Narrative synthesis, Systematic review

Anticipated or actual start date

14 December 2022

Anticipated completion date

01 April 2023

Funding sources/sponsors

CRUK

Conflicts of interest

Language

English

Country

England

Stage of review

Review Ongoing

Subject index terms status

Subject indexing assigned by CRD

Subject index terms

Anus Neoplasms; Carcinoma, Squamous Cell; Combined Modality Therapy; Epithelial Cells; Humans; Immunotherapy

Date of registration in PROSPERO

16 January 2023

Date of first submission

14 December 2022

Stage of review at time of this submission

The review has not started

Stage	Started	Completed
Preliminary searches	No	No
Piloting of the study selection process	No	No
Formal screening of search results against eligibility criteria	No	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

The record owner confirms that the information they have supplied for this submission is accurate and complete and they understand that deliberate provision of inaccurate information or omission of data may be construed as scientific misconduct.

The record owner confirms that they will update the status of the review when it is completed and will add publication details in due course.

Versions

16 January 2023

Appendix B – CORMAC-2 Protocol

International consensus to define outcomes for trials of chemoradiotherapy for anal cancer (CORMAC-2): Defining the outcomes from the CORMAC core outcome set

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Method Article

Keywords:

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Abstract

Introduction

Anal cancer is rare, but its incidence is increasing. Chemoradiotherapy is the primary treatment modality. Outcomes used in anal cancer trials vary which hinders evidence synthesis. Using a systematic review, patient interviews and a 2-stage Delphi consensus survey, the first CORMAC project brought together patients and healthcare professionals from across the world to agree shared priorities and make sure that studies of chemoradiotherapy treatments for anal cancer report outcomes that are meaningful to patients and health care professionals. CORMAC-1 established an internationally ratified core outcome set (COS) of 19 outcomes across 4 domains. These 19 outcomes are an agreed minimum that all clinical trials in chemoradiotherapy anal cancer trials should report. CORMAC-2 is the next phase which seeks to reach international agreement on the definitions for the 11 core outcomes in the domains of disease activity and survival. Agreeing definitions for these core outcomes will facilitate utilisation of the core outcome set, increasing outcome standardisation across trials thereby increasing the quality of data available for clinical decision-making and ultimately enhancing patient care.

Methods

The original CORMAC systematic review will be updated, focusing on 2 of the 4 COS domains, disease activity and survival domains. An international steering committee composed of international anal cancer trial experts will be formed. The committee will review the updated search results to develop a 2-stage Delphi consensus survey. The survey will be publicised through conferences, email lists, domestic and international bodies and will target healthcare and allied healthcare professional involved in the design, running, recruitment and publication of anal cancer trials. Following the 2-stage survey, a stakeholder meeting composed of the steering committee and selection of survey participants will ratify the results and agree a final set of core outcome definitions.

Ethics and dissemination

CORMAC-2 results will be disseminated through journal and conference publications to inform clinical teams and patient support groups to raise awareness and implementation of the core outcome set. Results will feed into the DECREASE study and it is registered with the Core Outcome Measures in Effectiveness Trials (COMET) initiative (1,2). As per the University of Manchester ethic decision tool, no ethical approval is required. Further information is available at https://cormacstudy.wordpress.com.

Introduction

Introduction

Anal cancers are rare, but the incidence is rapidly rising in the UK, Europe and the USA (3,4). Chemoradiotherapy is the primary treatment for patients with anal squamous cell carcinoma (ASCC) and treatment is associated with considerable short-term and long-term side effects. Five year survival is around for 76% for all stages of anal cancer, with considerable variation depending on stage at presentation (5). There are six Phase III randomised controlled trials that provide much of the evidence for this approach(6–11). Each of these six trials reported different primary outcomes and when the same outcome was used, the definition of these varied (12). These variations in reported outcomes and their definitions limits between-study comparisons (13), hinders evidence synthesis and reduces the improvement clinical research can have on patient care.

Core Outcome Research Measures in Anal Cancer (CORMAC) was an international patient and healthcare professional consensus study designed to address this issue. For clarity, CORMAC will be referred to as CORMAC-1 for the rest of the protocol. It led to the publication of an internationally ratified core outcome set (COS) for clinical trials of chemoradiotherapy interventions for ASCC (13). A COS is an agreed collection of outcomes that all trials looking at a specific clinical area report as a minimum. It is a recommendation of "what" should be reported (14,15). Agreement was reached for 19 outcomes across four domains: disease activity, survival, toxicity and life impact (Figure 1).

The aim of the CORMAC-2 project is to establish internationally agreed, standardised definitions for the 11 disease activity and survival outcomes in the CORMAC-1 COS (figure 1); appropriate measurement instruments for the outcomes in the toxicity and life impact domains will be identified in a separate piece of work. This will be achieved through an updated systematic review followed by an international consensus process involving a Delphi questionnaire and consensus meeting. Agreeing definitions for these core outcomes will facilitate utilisation of the core outcome set, increasing outcome standardisation across trials, thereby increasing the quality of data available for clinical decision-making and ultimately enhancing patient care.

Reagents

Equipment

Procedure

Methods

Agreement of the core outcome definitions will involve four packages across two phases. Phase 1 will involve collating existing outcome definitions and using these to construct questions for the next phase as well as advertising the project to the wider international anal cancer research community. Phase 2 will

involve an international online 2-stage Delphi questionnaire followed by a consensus meeting to review the results of the questionnaire and agree a final set of core outcome definitions. The methods used here are similar to the Definition for the Assessment of Time-to-event Endpoints in Cancer trials (DATECAN) initiative that have been used for various other cancer types including breast and pancreatic (16,17).

Project oversight

A steering committee has been assembled to oversee the project. Members include oncologists, colorectal surgeons and methodologists with leading roles in past and current anal cancer clinical trials and/or core outcome set development projects. Early engagement of international experts, especially those active in current clinical trials, will help to ensure validity of the Delphi questionnaire and facilitate wider international awareness and participation. The members of the steering committee are listed at the end of the document. The academic research language used to describe nuanced differences in survival and disease activity outcome definition is considered to be too technical to ask patients opinions within the Delphi questionnaire. Engagement with patient and public involvement (PPI) and advocacy groups will therefore be conducted separately and will focus on determining the impact and acceptability of modes of outcome measurement that are presented as options in the Delphi. For example, PPI groups will be asked about the impact and acceptability of different modalities and frequencies of assessment of treatment response. The Leeds Radiotherapy Research Group Public and Patient Involvement group and the Anal Cancer Foundation will provide patient representatives from the UK and USA respectively. Feedback from PPI groups will be summarised and presented to participants during the Delphi questionnaire and consensus meeting.

Phase 1

WP1: updated systematic review

As part of CORMAC-1, a systematic review of all outcomes reported in trials of chemoradiotherapy interventions for ASCC was undertaken (12). Where outcomes were defined, the definitions were recorded. Using the same search terms and inclusion/exclusion criteria, the systematic review will be updated to 11th February 2021 and used to collate lists of all the existing definitions for the disease activity and survival outcomes. This is to provide an updated comprehensive list of all possible definitions in the COS for disease activity and survival. Details of the systematic review including search strategy, eligibility and exclusion criteria can be found on PROSPERO (CRD42016036540).

WP2: Advertisement of survey

To maximise international utilisation of the outcome set, engagement from the wider international anal cancer research community is critical. The CORMAC-2 study and Delphi questionnaire will be promoted

and publicised at international conferences including IMAAC 2021 and ASCO GI 2022 as well as through active anal cancer trials networks such as PLATO (UK), IMAAC (international) and relevant subcommittees of NCRI (UK) and NROG (North America). Steering committee members will use their knowledge of local societies, meetings, email lists and contacts to increase participation of the questionnaire. Potential participants will be able to register their interest via the CORMAC website prior to the study opening. The study and Delphi questionnaire will be promoted and publicised through conferences, email lists, domestic and international bodies and will target healthcare and allied healthcare professional involved in the design, running, recruitment and publication of anal cancer trials.

Phase 2

WP3: Delphi process

Phase 2 will involve an international, two round online Delphi questionnaire. A Delphi process is a structured communication method designed to achieve consensus amongst a panel of experts or stakeholders. The aim of the Delphi process is to achieve consensus among stakeholders on definitions for the CORMAC-1 disease activity and survival outcomes.

The outcome definitions collated in WP1 will first be reviewed and options agreed by the steering committee. To achieve agreement, systematic review results, displaying how each core outcome in disease activity and survival outcomes has previously been defined and described will be discussed at an online meeting and opinions summarised. These options will be recirculated and rediscussed before the final set of options for definitions of each core outcome is agreed. These agreed options will form the basis of the Delphi questionnaire. Within the Delphi questionnaire, where definition options include complex criteria e.g. RECIST, detailed descriptions of the definitions will be provided as well as summarised information on where and how the definitions have previously been used.

The Delphi questionnaire will be run using DelphiManager software and administered in two sequential rounds. Participants will be asked to rate the appropriateness of definitions for each of the disease activity and survival outcomes on a Likert scale of 1 (very limited importance) to 9 (very high importance). Anonymised feedback of the summarised results of the previous round will be provided to participants before completion of the subsequent round. This process is intended to achieve consensus among participants by minimising the potential for bias towards the opinions of those who are more outspoken or whose views might be perceived as superior. The process will follow guidelines recommended by the COMET Minimum Standards in COS Development project (18). The Delphi questionnaire will be open to healthcare and allied healthcare professionals involved in the design, recruitment, running and publication of anal cancer research and trials. This will include:

Ø Clinical Oncologists

Ø Medical Oncologists

- Radiation Oncologists
- Radiologists
- Radiographers
- Pathologists
- Colorectal Surgeons
- Stoma nurses
- Gastroenterologists
- Radiophysicists
- Statisticians
- Trial managers

Information gathered from each participant will include:

- Discipline (medical oncologist, specialist nurse, etc).
- Involvement with trials (named author on publication of a trial of chemoradiotherapy in anal cancer; part of working group involved in a trial of chemoradiotherapy in anal cancer; part of working group for development of future trials in anal cancer).
- Country of practice

Instructions for how to complete the questionnaire will be included at the start of each round. Links to background reading regarding the definitions participants are scoring (for example the details of the RECIST criteria) will be available for participants for both rounds. Participants will also be able to suggest alternative outcome definitions for inclusion in round 2. Analysis of the first round will include summarising the scores for each of the definitions and collating the free text comments and alternative definitions provided. In round 2, participants will be shown the results from round 1, including their own round 1 score for each item and the summarised scores from other participants (as a histogram) as well as any alternative definitions suggested by R1 participants and relevant feedback from the free text responses (anonymised), and asked to consider this information before re-scoring each item. For each definition where PPI opinion has been given, a summary of this information will also be provided with the question.

Although the importance of completion of both rounds of the Delphi survey will be stressed to participants before commencing round 1, it is anticipated that some participants will drop out after the first round. Each participant will be ascribed a unique participant number when they sign up to complete round 1 enabling the identification of the attrition rate between rounds. This will allow the identification of participants who have completed both rounds, and analysis of whether participants who drop out before completion of round 2 appear to have views that are different to those who complete the process.

A clear definition of what constitutes consensus is essential to reduce potential bias in the interpretation of the results in favour of the opinions of the researchers. Consensus can be considered to have been reached if the majority of participants rank an outcome similarly. After the final round, we will assign each definition option to one of three categories:

1. Consensus in: 70% or more respondents rate the item as critically important (7–9) AND 15% or fewer rate the outcome as limited importance (1–3).

2. Consensus out: 50% or less of respondents rate the item as critically important (7-9).

3. No consensus

There are no universally agreed consensus criteria, and the criteria used here follow published recommendations. The consensus out definition has been adapted from CORMAC-1 (13), as it was found that very few participants rated outcomes as having limited importance (1-3).

WP4: consensus meeting

A consensus meeting will be held to discuss the results from the Delphi questionnaire and agree on a final set of definitions for publishing. Given the international nature of the steering committee it will be held online using videoconferencing software. The meeting will consist of a sample of Delphi participants and the steering committee. All participants registering to complete the Delphi process will be asked for their consent to be contacted about participation in the consensus meeting (tick box on registration page for Delphi).

Selection of individual definitions for each outcome will be based on the following:

1. If only 1 definition reaches the consensus in criteria, this will be recommended as the definition.

2. If more than 1 definition has reached consensus in criteria, these options will be discussed and voted on during the consensus meeting.

3. If no definition has reached consensus in criteria, the "consensus out" options will be discarded and options from the "no consensus" will be discussed.

4. If all options have reached the consensus out criteria, these options will be discussed.

The definitions that meet "consensus in" criteria (option 1 above) after the final round of the Delphi will be presented. These definitions will not be voted on again unless a fundamental problem with that definition is raised by consensus meeting participants. The remaining definitions (options 2, 3 and 4) will be presented and group discussion will be facilitated. This will be followed by anonymous voting using the same 9-point Likert scale and consensus criteria used in the Delphi Study. If a definition for an outcome is not agreed at the end of the first consensus meeting, subsequent meetings will be considered.

Troubleshooting

Time Taken

Anticipated Results

Ethics and dissemination

The benefits of COS are increasingly recognised by research funding bodies, regulators and journal editors, via the work of the COMET Initiative in promoting COS utilisation. The European Medicines Agency recommends COS use for clinical trials in asthma medicines (19) and the UK National Institute for Health Research (NIHR) recommends outcomes from established COS are included in any new trial proposal (20). CORMAC-2 results will be disseminated through journal and conference publications to inform clinical teams and patient support groups to raise awareness and implementation of the core outcome set. Results will feed into the DECREASE study (1) and is is registered with the Core Outcome Measures in Effectiveness Trials (COMET) initiative (2). As per the University of Manchester ethic decision tool, no ethical approval is required.

Further work is planned to define the measurement instruments required for appropriate assessment and definition of the core outcomes in the toxicity and life impact domains. Methods similar to the COnsensus-based Standards for the selection of health Measurement INstruments (COSMIN) checklist, that have been employed in other cancer types, will be used (21). Agreeing definitions for these core outcomes will facilitate utilisation of the core outcome set, increasing outcome standardisation

across trials thereby increasing the quality of data available for clinical decision-making and ultimately enhancing patient care.

Project team and steering group

Immediate project team

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Steering group

Richard Adams, Pragnan Das, Jennifer Dorth , Marianne Guren, Maria Hawkins, David Sebag-Montefiore.

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Acknowledgements

Figures

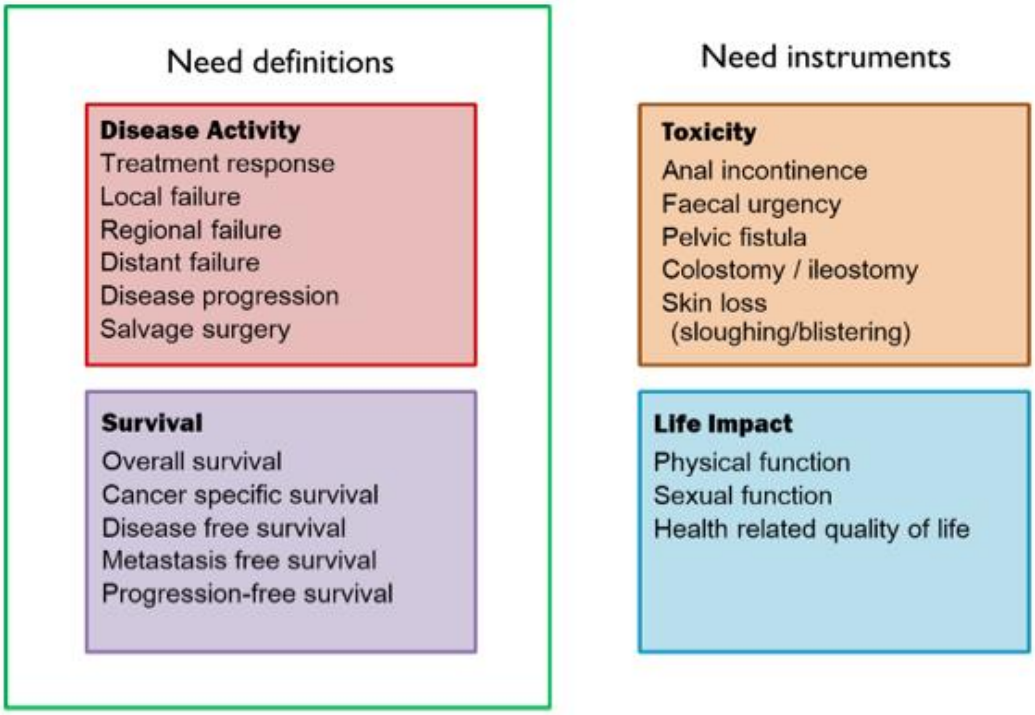

Figure 1

CORMAC-1 Core Outcome Set of 19 outcomes across 4 domains. Green box shows outcomes in need of agreed, standardised definitions which will be established through CORMAC-2