The assessment of T-cell subsets in response to treatment in patients with breast or oesophageal cancer

by

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Declaration of originality

I, Opope Oyaka Wedi, declare that this dissertation, which I hereby submit for the degree Masters of Science in medical immunology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

I am aware that, should the dissertation be accepted, I must submit the additional copies as required by the relevant regulations at least six weeks before the next graduation ceremony, and that the degree will not be conferred if this requirement is not fulfilled.

Signature…….. Date……

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Table of Contents

List of table **viii**

List of Figures x

Abbreviations xv

Abstract xvii

1 Introduction **1**

1.1 The immune system **4**

1.1.1 The innate immune system 5

1.1.2 The adaptive immune system

1.2 Antitumor immunity **15**

1.2.1 Carcinogenesis 15

1.2.2 Non-immunological surveillance 17

1.2.3 Immune surveillance 18

1.3 T regulatory cell **31**

1.3.1 Treg cell expression 31

1.3.2 Classification of Treg cells 33

1.3.3 T regulatory cell function 39

1.3.4 T regulatory cells and cancer 41

1.4 Antitumor treatment and immunity **43**

1.4.1 Surgical oncology 44

1.4.2 Chemotherapy 45

1.4.3 Radiotherapy 48

1.5 Breast cancer and immunology **50**

1.5.1 Breast cancer epidemiology 50

1.5.2 Pathology 52

1.5.3 Breast cancer prognosis 52
1.5.4 Breast cancer and T regulatory cells 58
1.6 Oesophageal cancer and immunology 62
1.6.1 Oesophageal cancer epidemiology 62
1.6.2 Pathology 62
1.6.3 Oesophageal cancer prognosis 63
1.6.4 Oesophageal cancer and T regulatory cells 64
2 Aim and objectives 68
2.1 Aim 68
2.2 Objectives 68
2.2.1 Primary objectives 68
2.2.2 Secondary objectives 68
3 Study cohort 70
3.1 Setting 70
3.2 Eligibility criteria 70
3.3 Breast cancer cohort 71
3.4 Oesophageal cancer cohort 72
3.5 Recruitment 73
4 Materials and methods 77
4.1 Flow cytometric analysis 77
4.2 Monoclonal antibodies 78
4.2.1 Intracellular staining 78
4.2.2 Extracellular staining 78
4.3 Gating strategy 79
4.3.1 Step 1: Identification of lymphocyte population 79
4.3.2 Step 2: Identification of total $CD4^+$ ($CD4^+CD45^+$) and total $CD8^+$ ($CD8^+CD45^+$) T- 80

lymphocyte populations

4.3.3 Step 3: Identification of $CD25^+$ expression by $CD4^+$ T-lymphocyte and $CD8^+$ T-lymphocyte 80

4.3.4 Step 4: Identification of $CD4^+$ and $CD8^+$ regulatory T-lymphocytes 80

4.4 Statistical analysis 85

5 T-lymphocyte subsets ($CD4^+$, $CD8^+$ and Treg cells) in breast and oesophageal cancer and association with stage of disease 86

5.1 Introduction 86

5.2 Results 86

5.2.1 Determination of stage of disease at first presentation in patients suffering from breast or oesophageal cancer 86

5.2.2 The prevalence of $CD4^+CD25^+$ and $CD8^+CD25^+$ T-lymphocyte cell population in breast and oesophageal cancer patients 90

5.2.3 $CD4^+$ and $CD8^+$ Treg cell populations identified 92

5.2.4 Association between circulating levels of $CD4^+$ and $CD8^+$ T-lymphocytes and pathological stage of breast cancer and oesophageal cancer 94

5.2.5 Association between circulating levels of $CD4^+$ and $CD8^+$ T-lymphocytes and pathological stage of breast cancer 96

5.2.6 Association between circulating levels of $CD4^+$ and $CD8^+$ T-lymphocytes and pathological stage of oesophageal cancer 98

5.2.7 Intracellular versus extracellular staining 100

5.3 Discussion 101

6 The evaluation of the relationship between $CD4^+$, $CD8^+$ and Treg cell populations and markers of prognosis in breast cancer: HER-2/neu and estrogen receptor status 106

6.1 Introduction 106

6.2 Results 107

6.2.1 HER-2/neu, estrogen receptor status and association with stage of disease 107
6.2.2 Evaluation of the immune profile, HER-2/neu and ER-receptor status 111
6.3 Discussion 131
7 Effect of antitumor treatment on T-lymphocyte distribution 136
7.1 Introduction 136
7.2 Results 138
7.2.1 Breast and oesophageal cancer: All antitumor treatment 138
7.2.2 Breast cancer patients: Surgical versus chemotherapeutic intervention 147
7.2.3 Oesophageal cancer patients 162
7.3 Discussion 171
8 Treatment outcome and association with T-lymphocyte immune profile 182
8.1 Introduction 182
8.2 Results 182
8.2.1 Detection of $CD4^+$ and $CD8^+$ T-lymphocyte populations in patients who survived compared to those who demised during the 5-7 week follow-up period 183
8.2.2 Detection of $CD4^+$ and $CD8^+$ Treg cell populations in patients who survived compared to those who demised during the 5-7 week follow-up period 186
8.2.3 Detection of $CD4^+$ and $CD8^+$ T-lymphocyte populations in patients who survived compared to those who demised during the 5-7 week follow-up period, stratified according to tumor type 190
8.3 Discussion 193
9 Conclusion 196
10 References 197
11 Ethical approval certificate 241

List of Tables

Table 1.1	$CD4^+$ T-lymphocyte sub-populations	12
Table 1.2	Histopathological types of invasive breast cancer	53
Table 1.3	Breast cancer metastatic determinants	55
Table 3.1	Clinicopathological characteristics of the patients	75
Table 6.1	Association between the general immune profile and HER-2/neu receptor status	113
Table 6.2	Association between the general immune profile and HER-2/neu receptor status, stratified according to the stage of disease	114
Table 6.3	Association between non-Treg immune profile and ER-receptor status	118
Table 6.4	Association between non-Treg immune profile and ER-receptor status, stratified according to stage of disease	119
Table 6.5	Association between immune profile and breast cancer tumor receptor status	128
Table 7.1	The $CD4^+$ and $CD8^+$ T-cell count (cells/µl) in breast and oesophageal cancer patients before treatment, at day 1 post-treatment and 5-7 weeks post-treatment	141
Table 7.2	The $CD4^+$ and $CD8^+$ T-cell count (cells/µl) in breast cancer patients receiving surgical or chemotherapeutic intervention: before treatment, at day 1 post-treatment and 5-7 weeks post-treatment	153
Table 7.3	The $CD4^+$ and $CD8^+$ T-cell count (cells/µl) in oesophageal cancer patients before treatment, at day 1 post-treatment and 5-7 weeks post-treatment	165

Table 8.1	The total $CD4^+$ T-lymphocyte, total $CD8^+$ T-lymphocyte count and $CD4^+$: $CD8^+$ T-cell ratio in breast and oesophageal cancer patients who survived and demised following antitumor treatment	184
Table 8.2	The $CD4^+$ and $CD8^+$ Treg cell count (cells/µl) in breast and oesophageal cancer patients who survived and demised, before receiving antitumor treatment and at day 1 post-treatment	187
Table 8.3	The $CD4^+$ Treg (%): total $CD4^+$ (%) and $CD8^+$ Treg (%): total $CD8^+$ (%) T-cell ratio in breast and oesophageal cancer patients who survived and demised, before treatment and at day 1 after treatment	188

List of Figures

Figure 1.1	Differentiation of naïve $CD4^+$ T-lymphocytes	11
Figure 1.2	Carcinogenesis	16
Figure 1.3	Schematic process illustrating immunoediting in cancer	20
Figure 1.4	Mechanisms used by malignant cells to evade antitumor immunity	24
Figure 1.5	T regulatory cell subsets	34
Figure 1.6	Differentiation of naïve $CD4^+$ T-cells into Treg cells and effector T-cells	35
Figure 1.7	Incidence of cancer in South Africa	51
Figure 4.3 A	Total lymphocyte population	81
Figure 4.3 B	Total $CD4^+$ T-lymphocyte and $CD8^+$ T-lymphocyte population	81
Figure 4.3 C	$CD25^+$ expression on $CD4^+$ T-lymphocyte populations	82
Figure 4.3 D	$CD25^+$ expression on $CD8^+$ T-lymphocyte populations	82
Figure 4.3 E	$CD4^+CD25^+CD127^+$ Treg cell populations	83
Figure 4.3 F	$CD8^+CD25^+CD127^+$ Treg cell populations	83
Figure 4.3 G	$CD4^+CD25^+FoxP3^+$ Treg cell populations	84
Figure 4.3 H	$CD8^+CD25^+FoxP3^+$ Treg cell populations	84
Figure 4.3 I	$CD4^+CD127^+FoxP3^+$ Treg cell populations	85

Figure 4.3 J	$CD8^+CD127^+FoxP3^+$ Treg cell populations	85
Figure 5.1	The distribution of breast and oesophageal cancer patients and stage of disease at presentation	89
Figure 5.2	The distribution of breast cancer according to race and stage of disease at presentation	90
Figure 5.3	The $CD4^+$ and $CD8^+$ T-lymphocyte subsets of breast and oesophageal cancer patients at first presentation	92
Figure 5.4	The distribution of circulating $CD4^+$ and $CD8^+$ T regulatory cell subsets of breast and oesophageal cancer patients at first presentation	94
Figure 5.5	The distribution of $CD25^+$ expression in $CD4^+$ and $CD8^+$ T-lymphocyte populations in breast and oesophageal cancer patients according to stage of disease progression at first presentation	96
Figure 5.6	The distribution of $CD25^+$ expression in $CD4^+$ and $CD8^+$ T-lymphocyte populations in breast cancer, according to stage of disease progression at first presentation	98
Figure 5.7	The distribution of $CD25^+$ expression in $CD4^+$ and $CD8^+$ T-lymphocyte populations in oesophageal cancer, according to stage of disease progression at first presentation	100
Figure 6.1	The percentage distribution of ER, PR and HER-2/neu receptor status in patients presenting with breast cancer	110
Figure 6.2	The relationship between receptor status and stage of disease, shown as a percentage distribution	111
Figure 6.3	The percentage distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in patients presenting with stage 1 and 2 breast cancer, stratified according to HER-2/neu receptor status	116
Figure 6.4	The percentage distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in patients	117

	presenting with stage 3 and 4 breast cancer, stratified according to HER-2/neu receptor status	
Figure 6.5	The $CD4^+$ and $CD8^+$ T-lymphocyte subsets in patients presenting with stage 1 and 2 breast cancer, stratified according to ER-receptor status, stage of disease and HER-2/neu receptor status	122
Figure 6.6	The $CD4^+$ and $CD8^+$ T-lymphocyte subsets in HER-2/neu positive breast cancer patients presenting with stage 3 and 4 disease, stratified according to ER-receptor status and stage of disease.	123
Figure 6.7	The $CD4^+$ and $CD8^+$ T-lymphocyte subsets in HER-2/neu negative breast cancer patients presenting with stage 3 and 4 disease, stratified along ER-receptor status and stage of disease.	124
Figure 6.8	The $CD4^+$ and $CD8^+$ Treg cell subsets in patients presenting with ER-positive stage 1 and 2 breast cancer, stratified according to HER-2/neu receptor status.	126
Figure 6.9	The $CD4^+$ and $CD8^+$ Treg cell subsets in patients presenting with HER-2/neu positive stage 3 and 4 breast cancer, stratified according to ER-receptor status.	127
Figure 6.10	The $CD4^+$ and $CD8^+$ Treg cell subsets in patients presenting with HER-2/neu negative stage 3 and 4 breast cancer, stratified according to ER-receptor status.	128
Figure 7.1	The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in breast and oesophageal cancer patients before treatment (red bar), at day 1 post-treatment (yellow bar) and 5-7 weeks post-treatment (blue bar)	140
Figure 7.2	The frequency distribution of $CD4^+$ and $CD8^+$ Treg subsets in breast and oesophageal cancer patients before treatment (brown bar), at day 1 post-treatment (green bar) and 5-7 weeks post-treatment (blue bar)	143
Figure 7.3	The frequency distribution of $CD4^+$ Treg (%): total $CD4^+$ (%) and $CD8^+$ Treg: (%) total $CD8^+$ (%) ratio in breast and oesophageal cancer patients before treatment, at day 1 after treatment and 5-7 weeks post-treatment	146

Figure 7.4	The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in breast cancer patients receiving chemotherapy and surgical intervention, before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bar)	150
Figure 7.5	The frequency distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in breast cancer patients receiving chemotherapy and surgical intervention, before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bar)	157
Figure 7.6	The frequency distribution of $CD4^+$ Treg (%): total $CD4^+$ T-cell (%) ratio in breast cancer patients receiving chemotherapy (green bars) and surgical (blue bars) intervention, before treatment, at day 1 after treatment and 5-7 weeks post- treatment	160
Figure 7.7	The frequency distribution of $CD8^+$ Treg (%): total $CD8^+$ T-cell (%) ratio in breast cancer patients receiving chemotherapy (blue bars) and surgical (red bars) intervention, before treatment, at day 1 after treatment and 5-7 weeks post- treatment	161
Figure 7.8	The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in oesophageal cancer patients before treatment (red bar), at day 1 post-treatment (yellow bar) and 5-7 weeks post-treatment (blue bar)	164
Figure 7.9	The frequency distribution of $CD4^+$ and $CD8^+$ Treg subsets in oesophageal cancer patients before treatment (brown bar), at day 1 post-treatment (green bar) and 5-7 weeks post-treatment (blue bar)	167
Figure 7.10	The frequency distribution of $CD4^+$ Treg (%): total $CD4^+$ T-cell (%) ratio and $CD8^+$ Treg (%): Total $CD8^+$ (%) T-cell ratio in oesophageal cancer patients, before treatment, at day 1 after treatment and 5-7 weeks post-treatment	170
Figure 8.1	The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment	186

Figure 8.2	The frequency distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment	189
Figure 8.3	The frequency distribution of $CD4^+$ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment	191
Figure 8.4	The frequency distribution of $CD8^+$ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment	192

Abbreviations

AFP	α-fetoprotein
AJCC	American Joint Committee on Cancer
APC	Antigen presenting cells
APC7	Anaphase promoting complex subunit 7
CD25-APC	Anti-human CD25-Allophycocyanin
APO-1	Apoptosis antigen 1
BCA	Breast cancer
CD	Cluster of differentiation
CI	Confidence interval
Conc.	Concentration
CTLA-4	Cytotoxic T-lymphocyte associated antigen 4
DN	Double negative
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
FasL	Fas ligand
FITC	Fluorescein Isothiocyanate
FoxP3	Foxhead box P3
GITR	Glucocorticoid-induced TNFR family-related receptor
IFN	Interferon
HER-2	Human epidermal growth factor 2 receptors
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen-D related
IARC	International Agency for Research on Cancer
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase enzyme
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
IPEX	Immune dysregulation polyendocrinopathy, enteropathy, X-linked syndrome
IQR	Interquartile range
MDSC	Myeloid derived suppressor cells
MHC	Major-Histocompatibility Complex
MICA/B	MHC class I chain-related proteins A and B molecule
MUC1	Mucin 1
N/A	Not applicable
NK1.1 receptor	Natural killer antigen 1.1
NK cell	Natural killer cell
NKG2D ligand	Natural killer group 2 member D
NKT	Natural killer T-cell
PB	Pacific Blue
PC7	Phycoerythrincyanin 7
PC5	Phycoerythrincyanin 5
PD-L1	Programmed death-I ligand
PE	Phycoerythrin
PGE	Prostaglandin E
PR	Progesterone receptor

ROS	Reactive oxygen species
SS	Side light-scatter
TCR	T- cell receptor
T_{FH}	Follicular helper T-cell
TGF	Transforming Growth Factor
Th cell	T-helper cell
TRAIL	TNF-related apoptosis-inducing ligand
TNF	Tumor necrosis factor
TNM	Tumor, Nodes, Metastases
TNMG	Tumor, Nodes, Metastases, Grade
Treg cell	Regulatory T-cell
SLE	Systemic lupus erythematosus
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

ABSTRACT

Background: Cancer is a crippling disease affecting 32.6 million people globally. It is currently ranked as the leading cause of death worldwide, and is associated with significant morbidity and mortality. Despite advances made in the prevention, detection and treatment of malignancy, there remains a dearth of accurate and feasible prognostic and predictive factors for use in the management of patients suffering from cancer in developing countries. It is widely recognized that the immune system plays a fundamental role in regulating the development and progression of malignancy. T-lymphocytes in particular have been categorized into T-cell populations which either promote (T regulatory cells) or prevent ($CD4^+$ and $CD8^+$ T-lymphocyte) cancer development and progression.

Aim: The aim of this thesis was to evaluate whether the levels of the respective pro- (T regulatory) and anti- ($CD4^+$ and $CD8^+$ T-lymphocytes) cancer T-lymphocyte subsets before treatment, and alterations of these levels following conventional anticancer treatment (surgery, chemotherapy and radiotherapy) could be used as markers of prognosis in conjunction with other well known prognostic factors, or as predictors of short term (5-7 weeks post-treatment) survival.

Methods: Blood samples were collected from 25 breast cancer, and 10 oesophageal cancer patients before antitumor treatment, at day 1 post-treatment and at 5-7 weeks post-treatment. The circulating concentrations and percentages of $CD4^+$, $CD8^+$ and T regulatory cells were determined from whole blood by flow cytometry. These levels were used to determine the

CD4: $CD8^+$ T-cell ratio, the $CD4^+$ Treg: total $CD4^+$ Treg ratio and the $CD8^+$ Treg: total $CD8^+$ Treg ratio.

Results: Early (stage 1 and 2) HER-2/neu negative breast cancer was associated with a higher $CD4^+$ (558 versus 133 cells/µl; P=0.05) and $CD8^+$ T-cell count (198 versus 58 cells/µl; P=0.05) as compared to early HER-2/neu positive breast cancer. Breast cancer patients showed a significant decline in $CD8^+CD25^+CD127^+$ Treg cell subsets (P=0.012) following antitumor treatment. However, oesophageal cancer patients showed an increasing trend in $CD8^+CD127^+FoxP3^+$ Treg cells at day 1 post-treatment (P=0.045) and at 5-7 weeks post-treatment (P=0.044). Patients who demised displayed a significantly lower $CD4^+$: $CD8^+$ T-cell ratio (1.2 versus 2.2; P=0.044) before treatment, as well as a lower $CD4^+$ (111 versus 390 cells/µl; P=0.0046) and $CD8^+$ T-cell count (88 versus 160 cells/µl; P=0.058) at day 1 post-treatment, as compared to those who survived. While patients who survived displayed a higher $CD8^+$ Treg: total $CD8^+$ T-cell ratio (1.48 versus 0.53 P=0.0126) before treatment, and a higher $CD4^+CD25^+CD127^+$ Treg cell level at day 1 post-treatment (9 versus 2 cells/µl; P=0.047), as compared to those who demised.

Conclusion

Taken together the findings of this thesis suggest that the $CD4^+$ and $CD8^+$ T-cell count and CD4:CD8 T-cell ratio may serve as predictors of short-term survival in the management of breast and oesophageal cancer. Furthermore, HER-2/neu receptor status may reflect the underlying immune status and antitumor effector activity of a patient. These findings also suggest that T regulatory cell subsets are a heterogeneous group of cells with variable frequencies at presentation and in responses to antitumor treatment depending on the cancer

type, stage of disease and antitumor treatment employed. This further illustrates the need for more work aimed at better characterizing the different Treg cell subsets to better identify which subsets are associated with patient prognosis. In summary, this work presents evidence to suggest a role for the T-cell immune profile as a prognostic and predictive parameter in the management of cancer. It argues for early assessments of cancer patients' immune status as part of the diagnostic work up and that the immune status is taken into consideration when decisions are made regarding treatment.

1 Introduction

Cancer is a crippling disease affecting an estimate 32.6 million people globally (IARC 2013). It is currently ranked as the leading cause of death worldwide, accounting for 13% of all deaths in 2008; and is associated with considerable morbidity and mortality (WHO 2013). During the past three decades, Africa has experienced an alarming rise in the prevalence of infectious diseases including human immunodeficiency virus (HIV), tuberculosis and malaria. These conditions account for seven times more deaths than cancer in sub-Saharan Africa and have been the main focus of international donors and health ministries in this region (Morhason-Bello et al. 2013). Although infectious diseases continue to plague the continent, recent reports suggest that the burden of disease attributable to cancer in sub-Saharan Africa is on the increase, with a projected 85% increase in the incidence of malignancy by 2030 (Bray et al. 2012). Furthermore, by the year 2050, 70% of the predicted 24 million people diagnosed with cancer will reside in low and middle-income countries (Parkin et al. 2001, Kingham et al. 2013).

The prognosis associated with malignancy in developing countries has been shown to be considerably worse than that observed in high-income settings (Ferlay et al. 2010). This has been attributed to a multitude of factors including; the shortage of healthcare professionals, poor infrastructure, advanced stage of disease at first presentation, limited treatment options, reliance on traditional medicine and poor treatment compliance (Kingham et al. 2013). Despite advances made in the prevention, detection and treatment of cancer on the African continent, this has not translated into improved prognosis (Kingham et al. 2013).

Over the past decades extensive research efforts have improved our understanding of the genetic, pathological and clinical components of malignancy. This information has been used

to direct clinical management and has facilitated the development of a wide variety of therapeutic interventions for patients suffering from cancer. However, despite the substantial progress made in cancer research, its immune-pathogenesis is still not completely understood. Furthermore, a large proportion of commonly used anticancer treatments are associated with considerable morbidity and even mortality due to their side effect profile (Esteva et al. 2004, Weigelt et al. 2005, Hayes et al. 2010). This is in addition to the fact that a number of these interventions fail to control the progression of the malignancy (Weigelt et al. 2005, Hayes et al. 2010). Irrespective of the clinical setting, the delicate and complex nature of cancer, as well as the broad spectrum of treatment options available makes the diagnosis and management of cancer extremely heterogeneous. This makes it imperative to have mechanisms that facilitate the selection of an appropriate therapeutic intervention for individual patients (Weigelt et al. 2005, Riley et al. 2009). These mechanisms should also facilitate the monitoring of treatment response, which would inturn enable clinicians to identify and respond to early signs of treatment failure.

Survival estimates and prognostic markers are essential in physicians' decision making, in all phases of illness (Christakis et al. 2000, Glare et al. 2005, Riley et al. 2009). Prognostic markers, defined as clinical measures used to estimate an individuals risk of future outcome (Riley et al. 2009) have been shown to enable the stratification of patients into different treatment groups and facilitate the counselling of oncology patients (Riley et al. 2009). Prognostic estimates in advanced cancer have also been shown to have increasing importance as a patient nears the end of life (Christakis et al. 2000). It is a time to formally re-evaluate the goals of treatment, which may change from life prolongation to palliation (Lamont et al. 2012).

Christakis and colleagues (2000) conducted a study which assessed the accuracy of doctors' prognostic estimates in 504 terminally ill patients, and found that only 20% of prognoses were accurate (Christakis et al. 2000). Although physicians have tried to provide their patients with a reasonable estimate of response to treatment and duration of survival, studies have shown that physicians are typically too optimistic in their estimates of patients' survival, with doctors overestimating survival by a factor of 5 (Christakis et al. 2000). These optimistic survival estimates have been associated with a delay in urgent hospice referrals and missed opportunities to devote more time to improving the quality of life of affected patients (Christakis et al. 2000). Furthermore, the physician's inaccurate prognostication may contribute to the perpetuation of the patients' misconception regarding the future and may result in patients making decisions which may have negative health outcomes (Christakis et al. 2000).

There is a growing body of evidence focusing on the identification of clinical (Glare et al. 2005), biochemical (Alcaraz et al. 2013) and histological (Nakamura et al. 2009) predictors of survival and tolerance to anticancer treatment, in addition to mechanisms whereby treatment response may be monitored (de Geus-Oei et al. 2009, Lilja et al. 2008). However, poor study design, lack of internal and external validity, inappropriate analysis and poor reporting have resulted in concerns regarding the validity of the results from numerous studies assessing prognostic markers (Altman et al. 1994, Altman et al. 1998, Kyzas et al. 2005). Commonly used modalities in our settings include patients' clinical signs and symptoms, performance status, biochemistry (e.g. calcium, C-reactive protein and α-fetoprotein levels), lymph node stage, histological grade and type, vascular invasion, tumour markers (AFP), menopausal status, tumour receptor status (HER-2/neu) and hormone receptor status (oestrogen-ER, and progesterone-PR) (Lamont et al. 2001, Glare et al. 2005,

Ray-Coquard et al. 2009, Watanabe et al. 2010, Hayes et al. 2010, Hurria et al. 2012). Other authors suggest prognostication models which incorporate information regarding the tumour (e.g. primary site, size, grade, tumour markers, metastatic sites etc.) and patient-related factors (demographics, co-morbidities, performance status, biochemistry, socio-economic status etc.) to give an individual patient a specific estimate of survival (Glare et al. 2005). Despite all these modalities we are still unable to accurately determine a patient's prognosis and monitor treatment response to anticancer treatment.

Recently there has been growing evidence that host defences against cancer play a significant role in predicting the clinical outcome of patients (Mafune et al. 2000). This has sparked a wave of interest in the role of the host's immune response against cancer, and the possibility of identifying immunological markers which may be used to monitor treatment response and reliably determine prognosis in patients presenting with cancer.

1.1 The immune system

The immune system is a vast and complex structure which protects the body against infectious agents and other harmful substances e.g. toxins. It is divided into two main components, namely the innate immune system and the adaptive immune system, which work in conjunction to perform four main functions, including (Murphy 2012):

- *Immunological recognition*: this enables cells of the immune system to recognize sources of infection within the human body;
- *Containment and eradication of infectio*n: immunological cellular and soluble factors with effector functions neutralize infective agents identified within the body;
- *Immune regulation*: the self-regulation of the immune system to ensuring that the body does not damage itself;

- *Immunological memory*: Following exposure to a pathogen, the immune system is able to mount a stronger and faster response to the same pathogen on subsequent exposures, thus rapidly and effectively eradicating the pathogen.

1.1.1 The innate immune system

Innate immunity comprises of a non-specific, cellular and biochemical defence mechanism including (Abbas et al. 2007):

- Physical and chemical barriers e.g. epithelial surface;
- Phagocytic cells e.g. neutrophils, macrophages;
- Natural killer (NK) cells;
- Blood proteins e.g. complement system, mediators of inflammation, cytokines.

Innate immunity can distinguish between normal tissues belonging to the organism (self) and newly encounter 'non-self' proteins or living cells thus serving as the first line of defence against pathogens (Bremers et al. 2000, Abbas et al. 2007). All "non-self" structures including malignant cells, viruses, bacteria etc. are recognized in a non-specific way and are attacked by non-specific effector cells (Bremers et al. 2000, Abbas et al. 2007). This ability to discriminate between tumor cells and normal cells demonstrates the crucial role which the innate immune system plays in the process of mounting an immune response against malignant cells (Dunn et al. 2004).

1.1.2 The adaptive immune system

In contrast to the innate immune system, the adaptive immune system comprises of antigen specific lymphocytes that mount a response against a wide variety of intracellular and extracellular pathogens encountered (Abbas et al. 2007, Murphy 2012). Adaptive immunity is characterized by the ability to increase in magnitude and defensive capability with each

successive exposure, and has been identified as a critical weapon in the quest for the solution to cancer (Seremet et al. 2011).

There are two main types of lymphocytes namely, B-lymphocytes and T-lymphocytes (Murphy 2012). All lymphocytes arise from common lymphoid progenitor cells which are found within the bone marrow. Precursor B-lymphocytes complete their maturation within the bone marrow and are morphologically similar to T-lymphocytes despite differences in protein production and function (Abbas et al. 2007, Ruffell et al. 2010, Murphy 2012). Following exposure to a foreign antigen, B-lymphocytes differentiate into plasma cells. These cells produce antibodies which function as soluble forms of B-cell receptors and mediate humoral (antibody-mediated) immunity (Abbas et al. 2007, Murphy 2012). The antigen specificity of the secreted antibodies is identical to their origin and facilitates the identification of pathogens by effector cells including T-lymphocytes (Abbas et al. 2007). As the focus of this thesis is on the role of T-lymphocytes in cancer, B-lymphocyte function and role in anticancer immunity will not be elaborated on.

1.1.2.1 T-lymphocyte maturation

T-lymphocytes also originate in the bone marrow from hematopoietic stem cells; however, the precursor cells migrate to the thymus via the blood stream where the process of maturation occurs. Once in the thymus the Notch1 receptor, found on T-lymphocyte precursor cells, receives a signal from thymic stromal cells, which instructs the cells to commit to the T-cell lineage (Murphy 2012b). This results in the precursor cells differentiating for a period of one week and then progressing into a phase of proliferation where $5x10^7$ new T-cells are produced daily (Murphy 2012b). These cells express CD2 receptors, but are regarded as double negative as they do not express any receptors which

define a mature T-lymphocyte, including, $CD4^+$, $CD8^+$ or $CD3^+$-T-cell receptor complex, they thus enter the double negative stage of differentiation (Lee et al. 2010, Murphy 2012b).

- **Double negative thymocytes**

Within the thymus, 5% of all thymocytes are regarded as double negative T-cells. At the beginning of the maturation process, this pool of double negative thymocytes comprises of double negative cells committed to becoming mature $CD4^+$ and $CD8^+$ T-lymphocytes, in addition to a mature T-cell population which has already arranged its T-cell receptor genes to encode for the γδ-T-cell receptor and are regarded as γδ-T-lymphocytes; as well as the Invariant natural killer T-lymphocyte population, which express αβ-T-cell receptors of limited variability and NK1.1 receptors (Lee et al. 2010, Murphy 2012b). The "double-negative" stage brings about the rearrangement of the immature thymocytes genes to eventually generate the T-cell receptor (TCR) α and β chains (Lee et al. 2010). The double negative stage is subdivided into 4 separate intervals including:

i. *Stage 1: Double Negative (DN) 1 thymocyte*

This period is characterized by the expression of Kit and $CD44^+$ on all double negative cells, with genes encoding for the αβ-chains of the TCR being in the germ line configuration (Murphy 2012b).

ii. *Stage 2: DN2 thymocyte*

Thymocytes begin to express CD25 (IL-2 receptor) and the TCR-β chain commences the process of rearrangement (Murphy 2012b).

iii. *Stage 3 and 4:DN3 thymocyte and DN4*

Thymocytes begin to lose the expression of Kit and CD44 ($CD44^{low}CD25^+$) and the rearrangement of the TCR-β chain continues. Once the β-chain gene has completed

the rearrangement, the thymocytes loose the expression of $CD25^+$ and progress to stage DN4. Cells which do not complete the rearrangement process remain at stage DN3 and soon die (Murphy 2012b). The expressed β chain combines with a pre-T-cell receptor-α chain to form a complete pre-T-cell receptor, which is expressed in conjunction with a CD3 molecule (CD3-pre-T-cell receptor complex) (Abbas et al. 2007B, Murphy 2012b) . Once the CD3-pre-TCR molecule has been assembled, the β-chain gene rearrangement is discontinued and the proliferation and differentiation of thymocytes into double positive cells begins ($CD3^+$-pre-$TCR^+CD4^+CD8^+$) (Abbas et al. 2007b, Murphy 2012B). At this stage thymocytes have now lost the expression of CD44 and CD25 (Murphy 2012b).

- **Double positive thymocytes**

Following this proliferative period the α-chain gene of the double-positive thymocyte undergoes rearrangement and completes the differentiation of the TCR-αβ receptor ($CD4^+CD8^+CD3^+$TCR-αβ) (Abbas et al. 2007B, Murphy 2012b). All lymphocytes contain a highly diverse TCR that enables the identification of almost any pathogen which enters the body (Abbas et al. 2007b, Murphy 2012b). The TCR-αβ facilitates the interaction between the T-lymphocytes and antigen presenting cells, thus initiating the process of inducing an antigen specific immune response (Abbas et al. 2007b).

- **Positive selection of thymocytes**

The double positive thymocytes have not been exposed to any antigenic stimulation, and consequently encounter epithelial cells within the thymic cortex, which display a variety of self-peptides bound to class I and II Major-Histocompatibility-Complexes (MHC). If the TCR

on a double positive cell recognizes the self-peptide being presented and concurrently, the $CD8^+$ receptor interacts with the class I MHC molecule, the T-lymphocyte will receive a signal that simultaneously prevents cell death and elicits the loss of $CD4^+$ receptor expression (Abbas et al. 2007b). It subsequently matures into class I MHC-restricted $CD8^+$ T-lymphocyte (Abbas et al. 2007b, Lee et al. 2010). A similar process occurs for the $CD4^+$ T-lymphocyte, resulting in a class II restricted $CD4^+$ T-lymphocyte (Abbas et al. 2007b). Cells which are not positively selected or whose TCR gene fails to complete rearrangement die (Lee et al. 2010).

- **Negative selection of thymocytes**

The single positive cells ($CD4^+$ or $CD8^+$ T-lymphocyte) then undergo a process of negative selection, which involves the exposure of T-lymphocytes to self-antigens derived from widely expressed proteins within the body (Murphy 2012b). T-lymphocytes which have a high reactivity to self-antigens are signalled to undergo apoptosis (Abbas et al. 2007b). Thus self reactive T-cells which are potentially dangerous to the body are destroyed (Abbas et al. 2007B, Murphy 2012b). Only two percent of cells which begin as double positive thymocytes and undergo this process of differentiation, maturation and screening over a 3 week period will eventually exit the thymus to form the peripheral T-cell repertoire (Murphy 2012b). At the end of the maturation and differentiation process 60% of cells which began as immature double negative thymocytes would have differentiated into mature T-lymphocytes expressing either $CD3^+$TCR-αβ$CD4^+$ or $CD3^+$TCR-αβ$CD8^+$ receptors (Murphy 2012b).

1.1.2.2 Cell mediated Immunity

Cell mediated immunity is a process characterized by the identification and processing of a pathogen-derived-antigen by an antigen presenting cell (APC), followed by the presentation of this processed antigen to a lymphocyte, which subsequently results in an immune response to the pathogen (Abbas et al. 2007b). T-lymphocytes are one of the mediators of cellular immunity, with the two major T-lymphocyte subsets being, helper T-lymphocytes ($CD4^+$ T-lymphocyte) and cytotoxic T-lymphocytes ($CD8^+$ T-lymphocyte) (Martorelli et al. 2010, Murphy 2012b) . These cells, in association with APCs, play a vital role in mounting an immune response against malignant cells.

- **$CD4^+$ T-lymphocytes**

$CD4^+$ T-lymphocytes are traditionally known as "helper cells" and have been shown to play a crucial role in the promotion of a variety of responses to different foreign antigens, in addition to the augmentation of antibody production by B-lymphocytes (Murphy 2012, Koretzky 2010). $CD4^+$ T-lymphocytes have also been shown to play a substantial role in antitumor immunity by eliminating malignant cells irrespective of the presence of the MHC class II complex, and facilitating $CD8^+$ T-lymphocyte antitumor response (Alcaraz et al. 2013). In the absence of an infection, T-lymphocytes are small featherless cells which circulate within the body with no functional activity and are termed "naïve T-lymphocytes" (Murphy 2012). Following exposure to an antigen, naïve $CD4^+$ T-lymphocytes may differentiate into several functional classes of effector cells with specific functions (Ruffell et al. 2010, Murphy 2012c) (Figure 1.1 and Table 1.1).

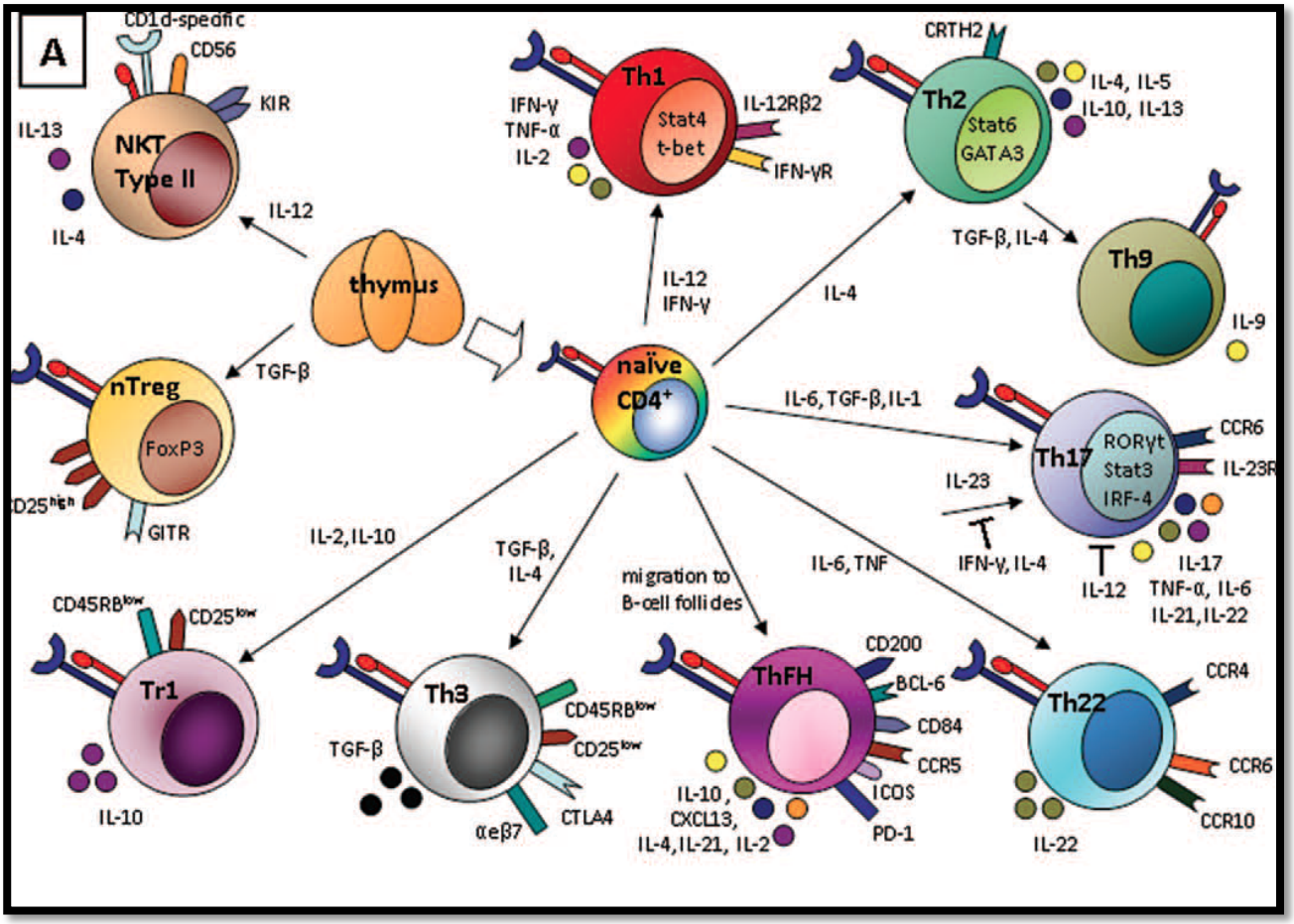

Figure 1.1: Differentiation of naïve $CD4^+$ T-lymphocytes (Martorelli et al. 2010)

Table 1.1: $CD4^+$ T-lymphocyte sub-populations

$CD4^+$ T-helper cell	Functions
Th1	Regulates delayed hypersensitivity reaction and cell mediated immunity to intracellular pathogens and tumor cells (Mosmann et al. 1996, Goto et al. 1999).
Th2	Controls allergy and parasitic infection e.g. helminthes by coordinating responses mediated by eosinophils, mast cells and IgE antibody isotype. It also helps coordinates humoral immunity and can be reprogrammed to differentiate into Th9 cells (Mosmann et al. 1996, Goto et al. 1999, Murphy 2012, Kaplan 2013).
Th3	Found predominantly within the mucosal immune system and controls the activation and suppression of the immune response in the mucosae (Murphy 2012 c).
Th9	Present in very low frequencies in humans but have been shown to exert an effect on mast cells, eosinophils, T-lymphocytes and epithelial cells. May play a role in autoantibody production in systemic lupus erythematosus (SLE) (Wong et al. 2010)
Th17	Protects the body against extracellular bacteria and fungal infection by stimulating neutrophil response. Coordinates tissue inflammation and autoimmunity (Murphy 2012 c)
Th22	Found in human skin and plays a role in skin homeostasis (Duhen et al. 2009)
T_{FH}	Migrates to B-cell follicles after activation and plays a role in B-cell activation and antibody production (Murphy 2012 c)

i. $CD4^+$ T-helper cell

This group of cells consists of a large number of T-helper subsets which may be differentiated according to their cytokine secretion profiles and functional properties (Martorelli et al. 2010). The main effector subsets including $CD4^+$ T-helper cell Type 1 (Th1), Th2, Th17 and Follicular helper T-cell (T_{FH}) with additional cell types identified including Th3, Th9 and Th22 (Staudt et al. 2010, Murphy 2012c, Basu et al. 2012). Despite the multiple roles these cells play in coordinating and facilitating an immune response against infective and non-infective pathogens, only Th1 has been shown to have a significant role in antitumor activity (Martorelli et al. 2010). Th1 regulates cell-mediated immunity against tumor cells by producing large quantities of interferon-γ (INF-γ), tumor necrosis factor-α (TNF- α) and IL-2 (Martorelli et al. 2010). The Th17 lineage has been identified fairly recently with an increased frequency of IL-17 producing cells in patients with prostate, ovarian and gastric cancer, with its presence in hepatocellular carcinoma patients being associated with poor prognosis (Ruffell et al. 2010). Studies have suggested that disequilibrium in circulating T regulatory cells and T_H17 cells may also promote the development of autoimmune diseases (Wong et al. 2010).

ii. $CD4^+$ T regulatory cells

This group's key function is to maintain homeostatic control and suppression of the immune response (Sakaguchi et al. 1995) . It has also been implicated in the pathogenesis of autoimmune disease as well as malignancy, reviewed by Taams et al (2006). This cell population will be discussed in more detail shortly.

iii. $CD4^+$ Type II natural killer T-cells

This cell also develops in the thymus and expresses the TCR-αβ receptor; however, they are characterized by a limited TCR repertoire and have the ability to recognize lipid epitopes presented by non-classical MHC molecules (CD1d) (Godfrey et al. 2007). These cells share characteristics of both NK cells and T-lymphocytes, and produce IFN-γ, TNF-α, IL-4 and IL-13, which direct a Th1 or Th2 polarized immune response (Ruffell et al. 2010). They are considered as part of the innate and adaptive immune system.

iv. Cytotoxic $CD4^+$ T-cells

This cell population was identified within the last decade as $CD4^+$ T-cells with granzyme B and perforin-containing granules (Martorelli et al. 2010). They normally represent a small proportion (2%) of the entire $CD4^+$ T-cell population in healthy people; however, these numbers increase in the presence of chronic viral infection with up to 50% of $CD4^+$ T-cells displaying cytotoxic potential in patients infected with HIV (Appay et al. 2002). These cells display immunophenotypic features consistent with antigen-experienced $CD4^+$ T-cells and end-stage differentiated T-cells (Appay et al. 2002). While $CD8^+$ T-lymphocytes have always been considered as the main cells capable of killing tumor cells, cytotoxic $CD4^+$ T-cells may present a good supplementary alternative since there are many factors which make cancer cells inherently resistant to $CD8^+$ T-cell antitumor activity including:

- The fact that malignant cells exhibit unpredictable antigen presenting capabilities and;
- The production of immunosuppressive substances by malignant cells (Martorelli et al. 2010).

Cytotoxic $CD4^+$ T-lymphocytes can assist by eradicating the cancer cells directly via presentation of tumor antigens on a MHC class II molecule or indirectly by the recognition of the tumor cell via an APC (Mumberg et al. 1999, Heller et al. 2006).

CD8$^+$ T-lymphocytes

CD8$^+$ T-lymphocytes have always been regarded as the more efficient and effective cell with regards to the eradication of virus infected cells and antitumor immunity (Martorelli et al. 2010). CD8$^+$ T-cells identify peptide-MHC class I complexes expressed by tumor cells and eradicate these malignant cells directly whilst, NK cells have been shown to regulate and facilitate CD8$^+$ T-lymphocyte function through the release of INF-γ (Dunn et al. 2004, Ruffell et al. 2010). Similar to the CD4$^+$ T-cells, there are numerous subtypes in the CD8$^+$ T-cell family including: type 1, type 2, type 17 CD8$^+$ effector T-lymphocytes and CD8$^+$ regulatory T-lymphocytes (Croft et al. 1994, Lu et al. 2008, Hamada et al. 2009).

1.2 Antitumor immunity

The immune system has been implicated in the pathogenesis of numerous medical conditions including cancer (Gallimore et al. 2008). Cancer arises from the uncontrolled proliferation of cells with the dispersal of clones of malignant cells (Abbas et al. 2007 c). Cancer growth is broadly determined by the ability of the cancer cells to evade non-immunological and immunological surveillance, the proliferative capability of the malignant cells and the ability of these cells to invade adjacent tissues locally as well as distally (Abbas et al. 2007 c, Kim et al. 2007).

1.2.1 Carcinogenesis

As stated by Devi and colleagues (2004), in 1948 Berenblum and Schubik described a multi-step process that disrupts normal cell regulation and results in the uncontrollable proliferation of rogue cells, which develop into solid tumors or haematological malignancies (Devi 2004). The development of cancer has been described in 3 phases including initiation, promotion and progression:

1.2.1.1 Phase 1: Initiation

During this stage, stable changes occur within the genome of somatic cells, naturally or following exposure to a carcinogen (e.g. viral infections, tobacco and alcohol) (Devi 2004). A proto-oncogene is regarded as a normal DNA sequence which following a mutation undergoes conversion to active oncogenes (American Cancer Society 2011) (Figure 1.2). This renders the affected cells and its progeny susceptible to neoplastic transformation (Devi 2004). These oncogenes may affect cellular behaviour, interrupt biochemical pathways that regulate cell proliferation and disturb normal development and differentiation of cells (United Nations Scientific Committee On The Effects Of Atomic Radiation 2000). For the oncogene to be expressed phenotypically, subsequent activation of multiple oncogenes need to occur, in addition to possible changes in the surrounding cellular environment (Devi 2004).

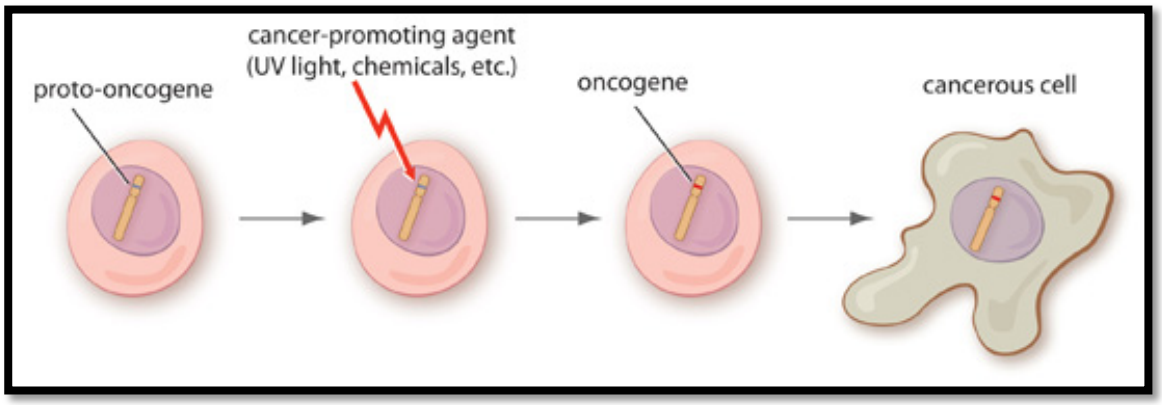

Figure 1.2: Carcinogenesis (Broad Institute of MIT 2014)

1.2.1.2 Phase 2: Promotion

Following the initiation of the somatic cell, phenotypic expression of the oncogenic mutation occurs as a result of exposure to both intracellular (e.g. additional oncogenic mutations) and extracellular (e.g. cytokines) factors (Devi 2004). These factors promote cellular growth and may disrupt regulatory signals that coordinate cell development and maintenance (Devi 2004).

1.2.1.3 Phase 3: Progression

During this stage the premalignant cells commit to neoplastic transformation and undergo further proliferation (Devi 2004). Consecutive changes and proliferation of the malignant cells result in the development of addition mutations, growth of the tumor in size and creation of a heterogeneous cluster of malignant cells within the tumor (Devi 2004). Subsequently, tumor progression advances to a point where malignant cells detach from the primary tumor mass and invade the adjacent tissue including the haematological and lymphatic system (Devi 2004). They are transported to other organs where they form secondary tumors.

1.2.2 Non-immunological surveillance

Non-immunological surveillance has been described as a process whereby host cells monitor and regulate tumor growth. This occurs either by:

- The identification of DNA damage by intracellular mechanisms which results in the arrest of cell growth and the induction of DNA reparation e.g. p53 pathway or;
- The induction of cell apoptosis following the identification of irreparable DNA damage or the activation of oncogenes (Kim et al. 2007).

These mechanisms work in conjunction with immunological mechanisms of tumor surveillance to prevent the development of malignancy (Kim et al. 2007).

1.2.3 Immune surveillance

As quoted by Khazaie and colleagues (2006), Paul Ehrlich was the first to introduce the concept of immune surveillance in 1909 when he proposed that, "the incidence of cancer would be much greater were it not for the vigilance of our immune defence system in identifying and eliminating nascent tumor cells" (Khazaie et al. 2006). Thereafter, Sir Burnet (1957) postulated that alterations in self-antigens, as a consequence of oncogenesis, may induce an immune response against the accumulating malignant cells (Burnet 1957). This would inturn culminate in the regression of the tumor, rendering the tumor clinically undetectable (Burnet 1957); however, the limitations in immunological research at the time prevented the validation of this hypothesis (Dunn et al. 2004). In the mid-20th century and early 1990's experimental evidence, including tumor transplantation models and knockout mouse models were used to validate the concept of immune surveillance against chemically (Old et al. 1962, Kaplan et al. 1998), virally (Klein et al. 1977) and spontaneously (Street et al. 2004, Smyth et al. 1999) induced tumors.

While experimental models have been extremely beneficial and have played a substantial role in the progress made in the field of tumor immunology, tumor development in immunocompromised mice have been difficult to interpret by virtue of the substantial defects imposed by each mutation (Smyth et al. 1999). This makes it difficult to translate this information into clinically relevant material, which can be used in a clinical setting for patient management. While it has long been recognised that patients suffering from congenital or acquired immune deficiencies are at an increased risk of developing malignancies (reviewed by Gatti et al. 1971), recent studies in the field of transplantation medicine has clearly demonstrated the importance of a functional immune system for anticancer activity. This comes following observations that immunocompromised organ transplant recipients

demonstrate an increased frequency of non-virally induced malignancies, which are not present in age-matched immunocompetent control patients (Sheil 1984, Birkeland et al. 1995, Hoshida et al. 1997, Jensen et al. 1999, Penn 2000, Adami et al. 2003, Dunn et al. 2004). These findings have further substantiated the concept of immune surveillance and suggest that, in the future, clinical modulation of the immune system could serve as an alternative form of therapeutic intervention to facilitate regression of an existing malignancy.

Originally, immune surveillance was described as a mechanism in which the adaptive immune system inhibited cellular transformation in the early stages of cancer (Dunn et al. 2004). However, studies have now demonstrated that host-protective mechanisms are instigated by both the innate and adaptive immune systems (Carey et al. 1976, Bauer et al. 1999, Shankaran et al. 2001), which in some instances protects the host from tumor development, while in other instances facilitates tumor progression and growth by selecting for malignancies with lower immunogenicity (Dunn et al. 2004). This led to the use of the phrase, ”immunoediting” instead of “immune surveillance” to describe the immune systems dual roles of preventing the onset of cancer and moulding the already formed tumor (Dunn et al. 2002). Immunoediting is described in 3 stages: elimination, equilibrium and escape (Figure 1.3).

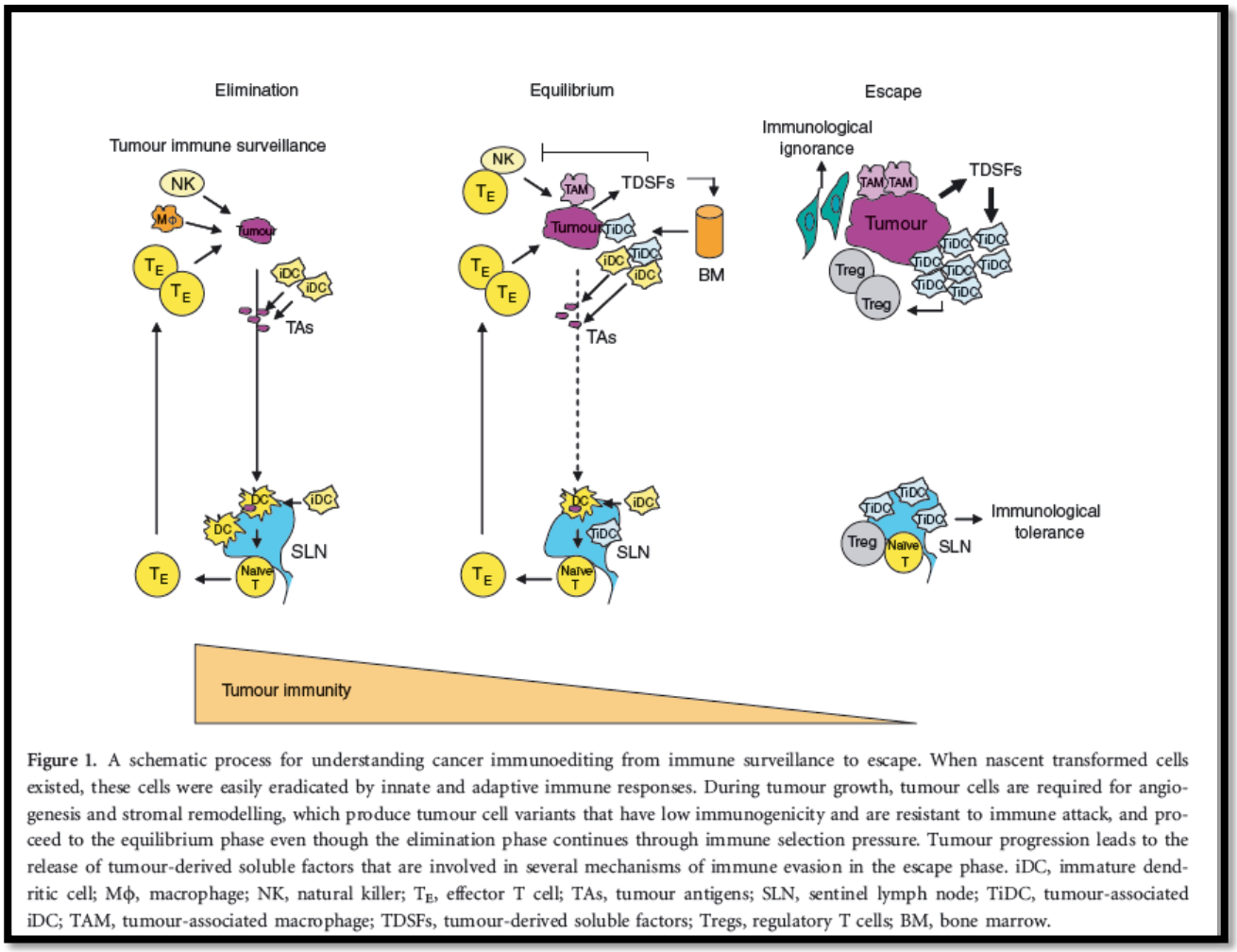

Figure 1. A schematic process for understanding cancer immunoediting from immune surveillance to escape. When nascent transformed cells existed, these cells were easily eradicated by innate and adaptive immune responses. During tumour growth, tumour cells are required for angiogenesis and stromal remodelling, which produce tumour cell variants that have low immunogenicity and are resistant to immune attack, and proceed to the equilibrium phase even though the elimination phase continues through immune selection pressure. Tumour progression leads to the release of tumour-derived soluble factors that are involved in several mechanisms of immune evasion in the escape phase. iDC, immature dendritic cell; Mϕ, macrophage; NK, natural killer; T_E, effector T cell; TAs, tumour antigens; SLN, sentinel lymph node; TiDC, tumour-associated iDC; TAM, tumour-associated macrophage; TDSFs, tumour-derived soluble factors; Tregs, regulatory T cells; BM, bone marrow.

Figure 1.3: Schematic process illustrating immunoediting in cancer (Kim et al. 2007).

1.2.3.1 Stage 1: Elimination

This stage refers to the original concept of immune surveillance and entails the eradication of developing malignant cells (Kim et al. 2007). This requires a coordinated response by both the innate and adaptive components of the immune system (Kim et al. 2007). Following neoplastic transformation, tumor cell proliferation commences and continues until the tumor mass reaches a measurement of 2-3mm. This is followed by the initiation of angiogenesis and

invasion of the local tissue for tumor growth to continue (Hanahan et al. 1996, Kim et al. 2007). The stromal remodelling promotes the release of proinflammatory molecules that alert the innate immune system to the presence of malignant cells (Dunn et al. 2002). Tumor cells have also been shown to release chemokines (e.g. CXCL-9, CXCL-10), which together with the proinflammatory molecules summon the cellular components of the innate immune system, including NK cells, NKT-cells, γδT-cells and macrophages to the site of malignancy (Dunn et al. 2002, Kim et al. 2007).

The effector cells of the innate immune system initiate the process of eliminating the malignant cells directly via the; Perforin, TNF-related apoptosis-inducing ligand (TRAIL) and FasL dependent mechanisms, production of tumoricidal products, and indirectly through the production of IL-12 and IFN-γ (Coughlin et al. 1998, Smyth et al. 2001, Takeda et al. 2001, Dunn et al. 2004). The production of IFN-γ contributes significantly to antitumor progression by promoting anti-proliferative (Bromberg et al. 1996), pro-apoptotic (Kumar et al. 1997) and angiostatic effects (Luster et al. 1993, Coughlin et al. 1998, Qin et al. 2000), in addition to facilitating the activation of tumor infiltrating macrophages and NK cells (Dunn et al. 2004). The release of IL-12 by macrophages stimulates the NK cells further, which in turn results in the release of larger quantities of INF-γ (Dunn et al. 2002).

The apoptosis of tumor cells results in the liberation of tumor-specific antigens (Dunn et al. 2004). NK cells promote the maturation of circulating dendritic cells, with additional activation occurring post exposure to proinflammatory molecules and cytokines released during the initial phase of the antitumor immune response (Gerosa et al. 2002). The liberated tumor antigens undergo phagocytosis by dendritic cells, where they are processed and transported to the draining lymph nodes and secondary lymphoid organs, where they are

presented to naive $CD4^+$ T-lymphocytes and $CD8^+$ T-lymphocytes (Kim et al. 2007), resulting in the maturation of these cells.

Tumor specific $CD4^+$ T-lymphocytes and $CD8^+$ T-lymphocytes migrate to the primary tumor site and attack and, eradicate immunogenic malignant cells (Kim et al. 2007). The production of IL-2, by $CD4^+$ T-lymphocytes, and IL-15 by host cells, facilitates the maintenance of $CD8^+$ cytotoxic T-lymphocyte function and viability (Dunn et al. 2004). Tumor specific $CD8^+$ T-cells play a crucial role in the eradication of the malignant cells by lysing tumor cells via death cell ligands e.g. TRAIL, granzyme/perforin pathways as well as producing large quantities of INF-γ, which in turn contributes to INF-γ led tumorolysis (Dunn et al. 2004, Töpfer et al. 2011).

$CD4^+$ T-lymphocytes have also demonstrated additional non-redundant antitumor effector activity, independent of the $CD8^+$ T-lymphocytes (Pardoll et al. 1998, Töpfer et al. 2011). They play a central role in the initiation and maintenance of antitumor response with Th1 and Th2 effector pathways eliminating tumors through the activation and recruitment of effector cells including macrophages and eosinophils (Pardoll et al. 1998). Following the demonstration of the essential role of $CD4^+$ T-lymphocytes and $CD8^+$ T-lymphocytes in antitumor immunity, studies demonstrated that the peripheral blood $CD4^+$: $CD8^+$ T-cell ratio could be used as an indicator of patients' immunity (Mafune et al. 2000). These studies demonstrated that a progressive decrease in both circulating $CD4^+$ and $CD8^+$ T-lymphocytes was associated with the spread of disease in patients with breast, malignant melanoma, lung and colorectal cancers (Blake-Mortimer et al. 2004).

1.2.3.2 Stage 2: Equilibrium

During the elimination period T-lymphocyte and INF-γ associated selection pressure results in the survival of variants of malignant cells with increased resistance to the antitumor immune response (Dunn, Old et al. 2004). A state of equilibrium is attained whereby effector cells of the immune system continually destroy immunogenic malignant cells, while new variants of genetically unstable tumor cells arise carrying mutations rendering them resistant to a competent immune system (Kim et al. 2007). This process continues for numerous years and these resistant variants ultimately progress to clinically detectable malignancies (Loeb et al. 2003).

1.2.3.3 Stage 3: Escape

In the escape phase, malignant cells which survived the elimination and equilibrium phase have accumulated genetic mutations which grant them resistance to attacks by the immune system, thus allowing the tumor to grow to clinically detectable volumes without immunological threat (Dunn et al. 2004). The tumor cells employ numerous evasive strategies including tumor related mechanisms and immunosuppressive cells (Figure 1.4) (Dunn et al. 2002, Kim et al. 2007, Morse et al. 2009, Töpfer et al. 2011).

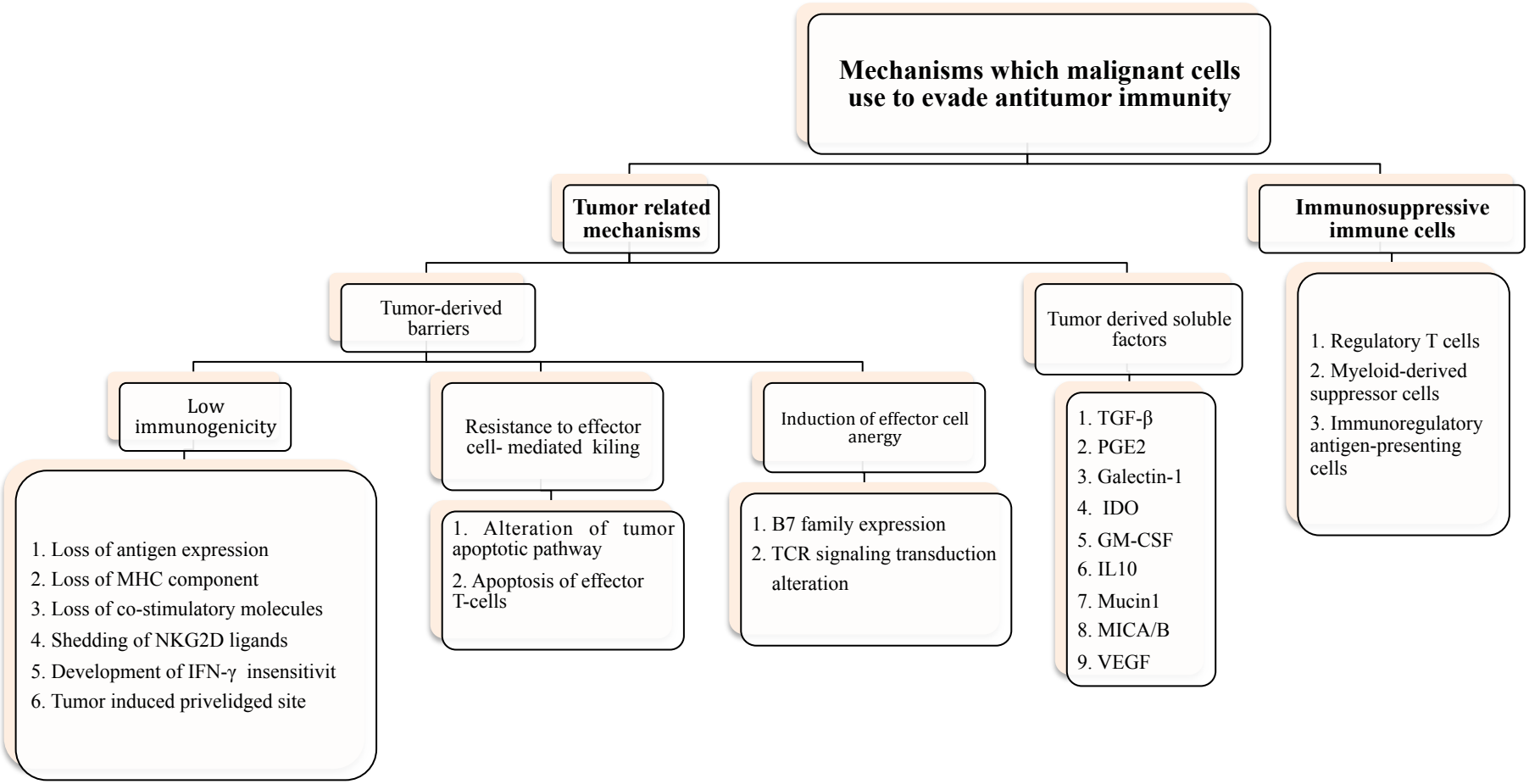

Figure 1.4: Mechanisms used by malignant cells to evade antitumor immunity. (Reichert et al. 1998, Marincola et al. 2000, Bellamy et al. 2001, Dong et al. 2002, Groh et al. 2002, Webb et al. 2002, Terabe et al. 2003, Parry et al. 2005, Orentas et al. 2006, Kim et al. 2007, Mourad-Zeidan et al. 2008, Rodríguez et al. 2008, Shevach et al. 2008, Morse et al. 2009, de Koning et al. 2011, Töpfer et al. 2011, Murphy 2012d)

Tumor related mechanisms (Figure 1.4)

I. Tumor-derived barriers

i. Low immunogenicity

One of the mechanisms employed by malignant cells, to evade the immu
by the reduction of their immunogenicity (Murphy 2012d). The malign
conceal themselves from antitumor effector cells by modulating the tumor
within the tumor microenvironment through the creation of a physical
prevents the interaction of immune cells and the tumor (Murphy 2012d).
mediated through the secretion of materials e.g. collagen, and surroundi
non-tumor cells including fibroblasts, endothelial cells and extracellular m
al. 2007, Murphy 2012d). The fibroblasts and endothelial cells compete wi
dendritic cells for tumor antigens while the extracellular matrix bind tu
(Murphy 2012d). This process is known as immunological ignorance. Th
conjunction to additional mechanisms, which renders the tumor undetect
T-lymphocytes and NK cells, including the down-regulation or loss of
MHC class 1 molecules, tumor-antigen expression, co-stimulatory m
family, CD40, CD80, HLA-DR) and NKG2D ligands (Marincola et al. 2
al. 2002, Groh et al. 2002, Rodríguez 2008, Murphy 2012d). Lastly, tun
induce the down-regulation or dysfunctional expression of adhesion
blood vessels e.g. addressin, E-selectin and intracellular adhesion molecu
I), which results in impaired extravasation of T-lymphocytes at the tumor
al. 2008).

ii. Resistance to effector cell-mediated killing

- *Apoptosis of effector cells:* This mechanism is mediated by 2 groups of receptors including Fas/Fas ligand and programmed death-1 ligand (PD-L1/PD-H1). Fas receptor, which is also known as apoptosis antigen 1 (APO-1) or CD95, is a member of the TNF receptor family and are highly expressed on effector T-lymphocytes following activation in the absence of co-stimulatory molecules (Morse et al. 2009, Töpfer et al. 2011). Fas ligands (FasL) are over-expressed in tumor cells, which initiate the apoptosis of effector T-cells following interaction with the Fas receptor on the T-cell (Webb et al. 2002). Programmed death ligands interact with Fas or PD-1 receptors on activated T-lymphocytes and NK cells (Morse et al. 2009).

- *Alteration of tumor apoptotic pathways:* One of the mechanisms which $CD8^+$ T-cells, NK cells and NKT-cells use to eliminate malignancy is via the perforin-dependent cell death pathway (de Koning et al. 2011). However, tumor cells have been shown to evade this mechanism through the interference of perforin/granzyme signaling pathway via the expression of cell death inhibitors including caspase-inhibitors XIAP and FLIP, as well as granzyme B inhibiting serine protease inhibitor, SERPINB9 (de Koning et al. 2011, Töpfer et al. 2011). Tumor cells have also been shown to evade the immune response through the down-regulation or inactivation of death receptors at the transcriptional level, as well as the expression of decoy transmembrane or soluble receptors with truncated non-functional or missing death domains (Peli et al. 1999, Töpfer et al. 2011).

iii. Induction of effector cell anergy

- *B7 family expression*: Tumor cells have been shown to express B7 family receptors, which interact with inhibitory co-receptors Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and PD-1 (Zou et al. 2008). CTLA-4 interaction results in decreased IL-2 production, impaired TCR signaling and cell cycle arrest (Parry et al. 2005, Hodi 2007). PD-1 is up regulated by IFN-γ (Blank et al. 2004).

- *TCR signal transduction alteration*: TCR/CD3-ζ signal is one of 3 essential signals required for Th1 polarization, T-lymphocyte proliferation, tumor antigen expression and successful tumor response (Kim et al. 2007). Tumor induced myeloid derived suppressor cells (MDSC) have been shown to deplete the extracellular milieu of L-arginine, which leads to the inhibition CD3-ζ chain expression of the TCR-CD3 receptor complex and subsequently induces the arrest of T-cell proliferation (Rodríguez et al. 2008). The loss of CD3-ζ chain in tumor infiltrating lymphocytes is also associated with the down-regulation of INF-γ and an increase in TGF-β and IL-10 cytokine levels (Kim et al. 2007). This alteration has been noted in numerous tumors including, oral cancer (Reichert et al. 1998), pancreatic cancer (Schmielau et al. 2001) and ovarian cancer (Lockhart et al. 2001) with studies suggesting a possible associations to poor prognosis. Furthermore, TCR/CD3-ζ receptor is also considered as a caspase 3 substrate, which tumor cells may utilize to trigger apoptosis of effector T-lymphocyte (Gastman et al. 1999, Gastman et al. 2000).

II. Tumor-derived soluble factors

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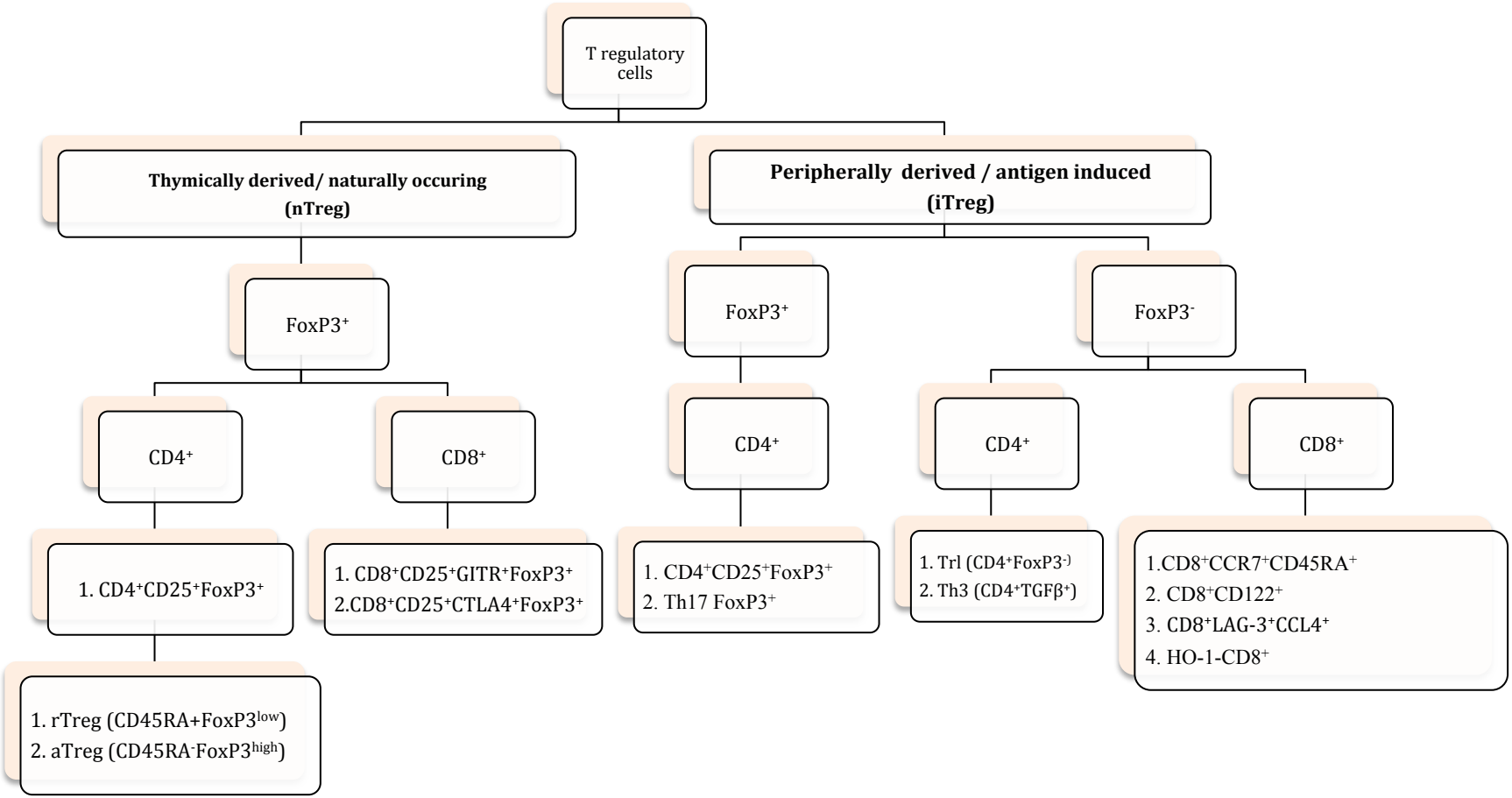

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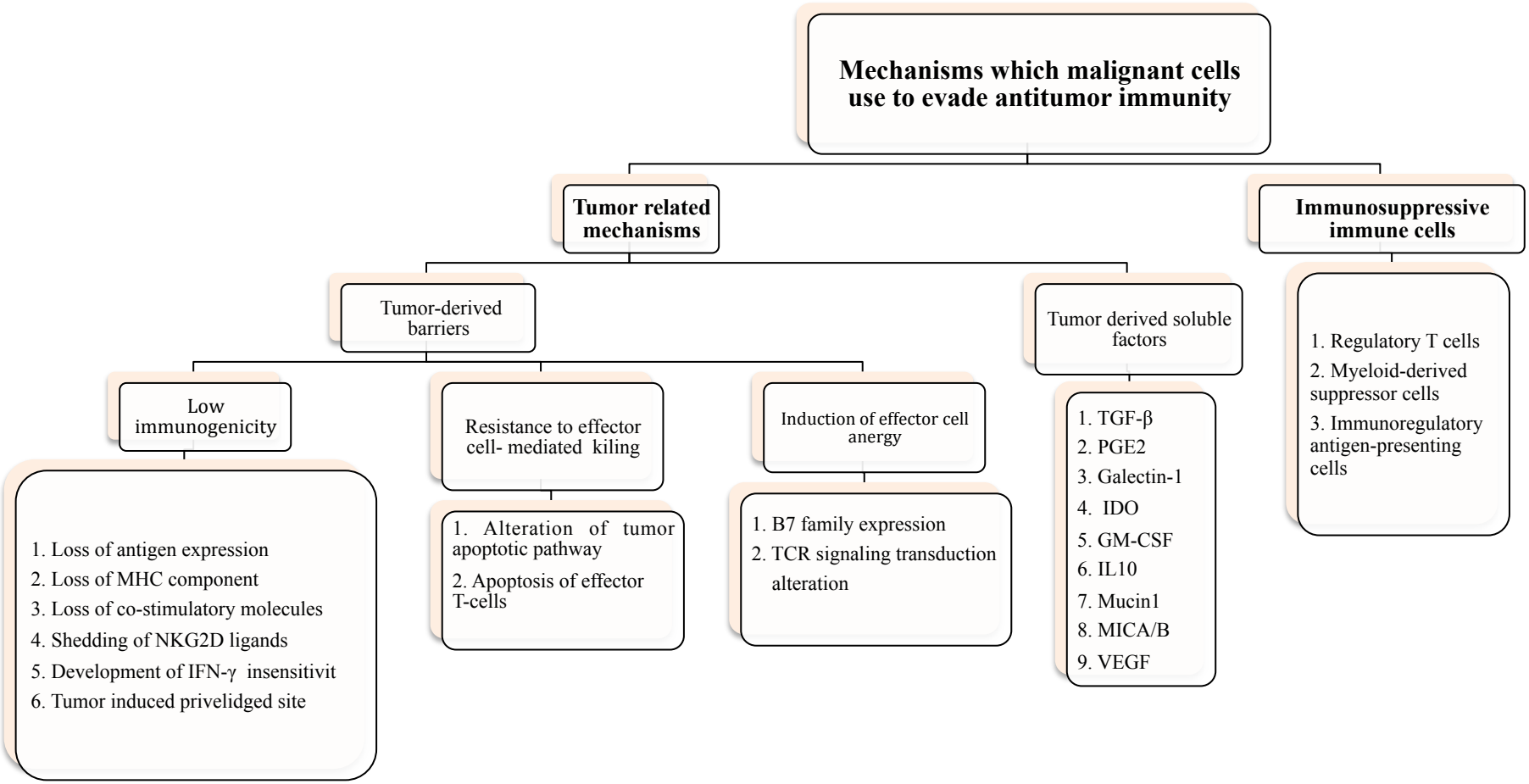

Figure 1.4: Mechanisms used by malignant cells to evade antitumor immunity. (Reichert et al. 1998, Marincola et al. 2000, Bellamy et al. 2001, Dong et al. 2002, Groh et al. 2002, Webb et al. 2002, Terabe et al. 2003, Parry et al. 2005, Orentas et al. 2006, Kim et al. 2007, Mourad-Zeidan et al. 2008, Rodríguez et al. 2008, Shevach et al. 2008, Morse et al. 2009, de Koning et al. 2011, Töpfer et al. 2011, Murphy 2012d)

Tumor related mechanisms (Figure 1.4)

I. Tumor-derived barriers

i. Low immunogenicity

One of the mechanisms employed by malignant cells, to evade the immu
by the reduction of their immunogenicity (Murphy 2012d). The malign
conceal themselves from antitumor effector cells by modulating the tumor
within the tumor microenvironment through the creation of a physical
prevents the interaction of immune cells and the tumor (Murphy 2012d).
mediated through the secretion of materials e.g. collagen, and surroundi
non-tumor cells including fibroblasts, endothelial cells and extracellular m
al. 2007, Murphy 2012d). The fibroblasts and endothelial cells compete wi
dendritic cells for tumor antigens while the extracellular matrix bind tu
(Murphy 2012d). This process is known as immunological ignorance. Th
conjunction to additional mechanisms, which renders the tumor undetect
T-lymphocytes and NK cells, including the down-regulation or loss of
MHC class 1 molecules, tumor-antigen expression, co-stimulatory m
family, CD40, CD80, HLA-DR) and NKG2D ligands (Marincola et al. 2
al. 2002, Groh et al. 2002, Rodríguez 2008, Murphy 2012d). Lastly, tun
induce the down-regulation or dysfunctional expression of adhesion
blood vessels e.g. addressin, E-selectin and intracellular adhesion molecu
I), which results in impaired extravasation of T-lymphocytes at the tumor
al. 2008).

ii. Resistance to effector cell-mediated killing

- *Apoptosis of effector cells:* This mechanism is mediated by 2 groups of receptors including Fas/Fas ligand and programmed death-1 ligand (PD-L1/PD-H1). Fas receptor, which is also known as apoptosis antigen 1 (APO-1) or CD95, is a member of the TNF receptor family and are highly expressed on effector T-lymphocytes following activation in the absence of co-stimulatory molecules (Morse et al. 2009, Töpfer et al. 2011). Fas ligands (FasL) are over-expressed in tumor cells, which initiate the apoptosis of effector T-cells following interaction with the Fas receptor on the T-cell (Webb et al. 2002). Programmed death ligands interact with Fas or PD-1 receptors on activated T-lymphocytes and NK cells (Morse et al. 2009).

- *Alteration of tumor apoptotic pathways:* One of the mechanisms which $CD8^+$ T-cells, NK cells and NKT-cells use to eliminate malignancy is via the perforin-dependent cell death pathway (de Koning et al. 2011). However, tumor cells have been shown to evade this mechanism through the interference of perforin/granzyme signaling pathway via the expression of cell death inhibitors including caspase-inhibitors XIAP and FLIP, as well as granzyme B inhibiting serine protease inhibitor, SERPINB9 (de Koning et al. 2011, Töpfer et al. 2011). Tumor cells have also been shown to evade the immune response through the down-regulation or inactivation of death receptors at the transcriptional level, as well as the expression of decoy transmembrane or soluble receptors with truncated non-functional or missing death domains (Peli et al. 1999, Töpfer et al. 2011).

iii. Induction of effector cell anergy

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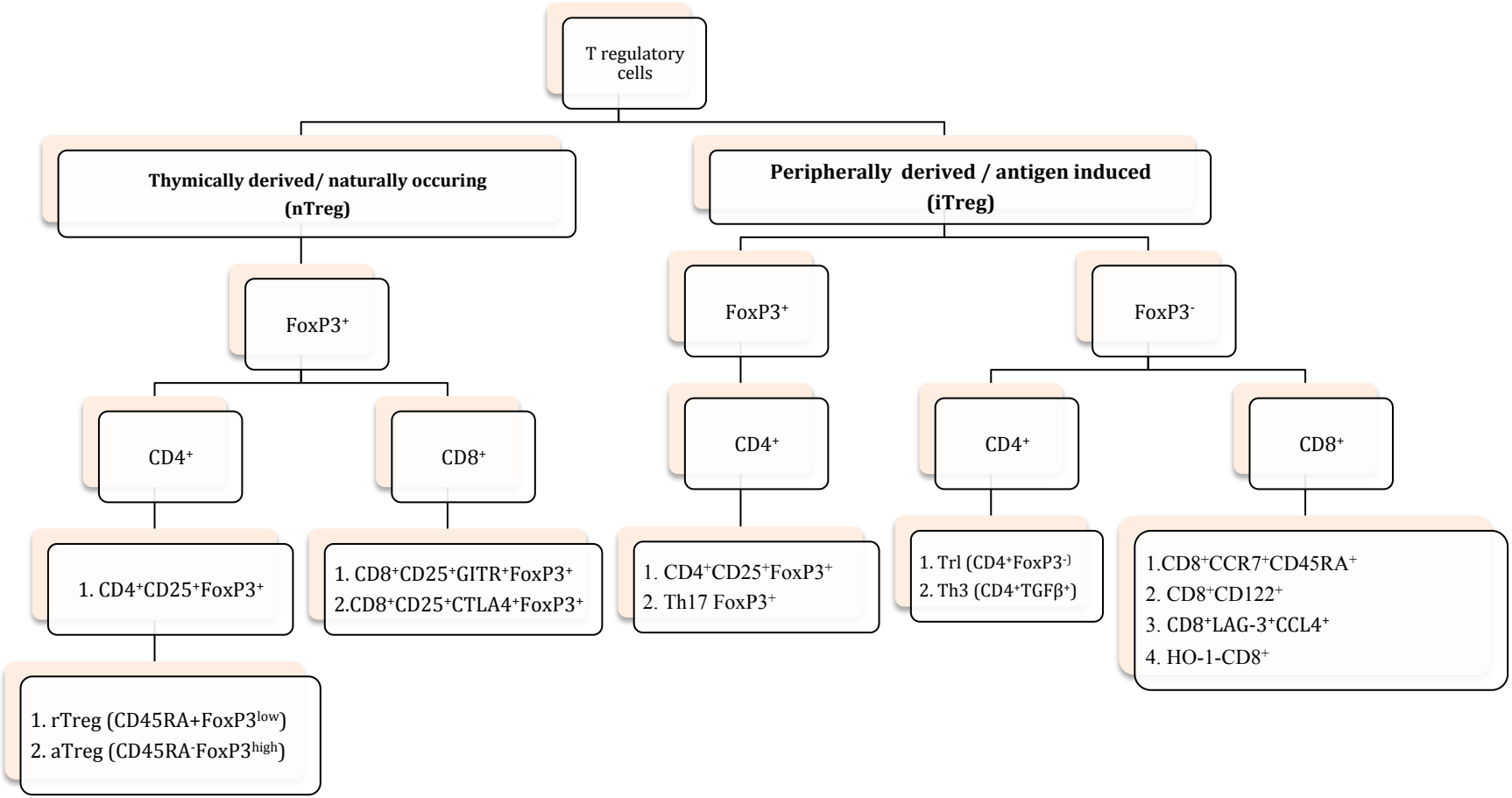

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1.3.2.1 CD4$^+$ Treg cells

CD4$^+$ Treg cells consists of numerous subsets including thymically derive CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (Fontenot et al. 2003), antigen-induce CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (Zheng et al. 2007) and CD4$^+$FoxP3$^-$ Trl cel 1.6) (Miyara et al. 2009, Grazia et al. 2006).

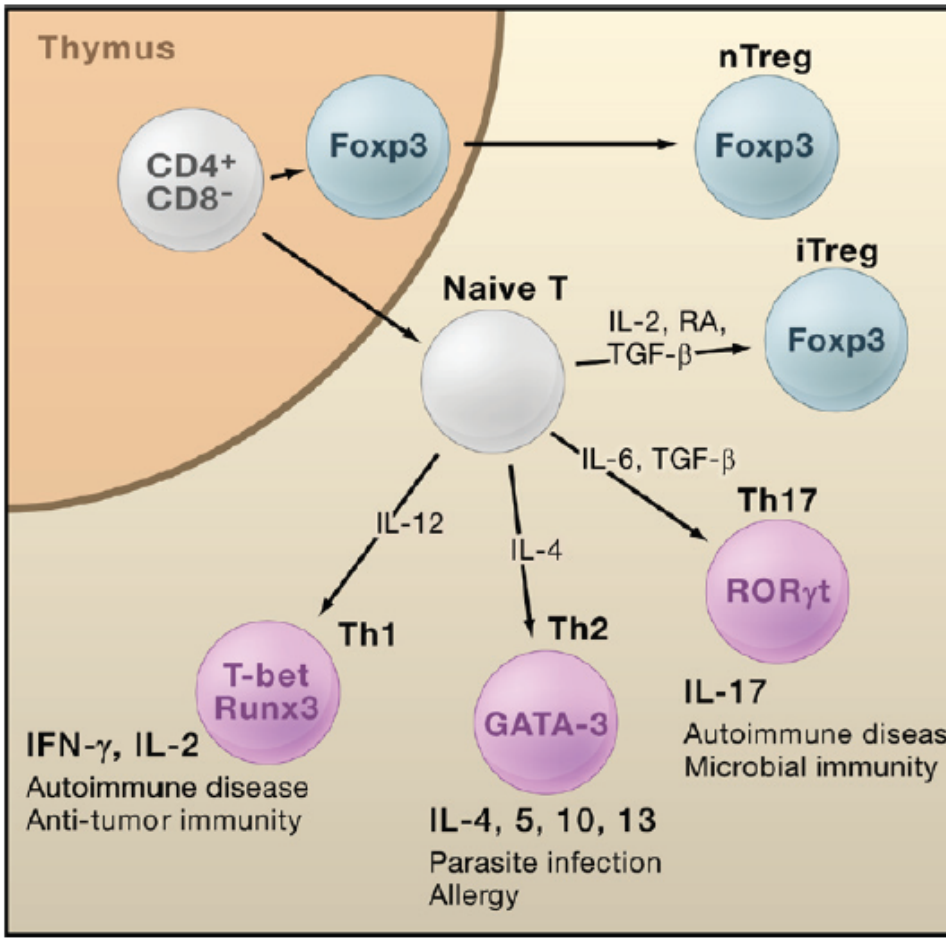


Figure 1.6: Differentiation of naïve CD4$^+$ T-cells into Treg cells and effect (Sakaguchi et al. 2008)

i. *Thymically derived Treg cell ($CD4^+CD25^+FoxP3^+$):* These cells arise through a process of central tolerance and migrate to the periphery where they induce effector suppression by a cell-to-cell dependent contact mechanism without specific antigen stimulation (Sakaguchi et al. 2006).

ii. *Antigen-induced Treg cell ($CD4^+CD25^+FoxP3^+$):* These cells arise following the exposure of $CD4^+$ $CD25^-$ naïve T-cells and effector T-lymphocytes to a suppressive cytokine environment (Zheng et al. 2007) or following an encounter with naturally occurring $CD4^+CD25^+FoxP3^+$ Treg cells (Qiao et al. 2007). These cells mediate immune suppression through a cell-to-cell contact mechanism, as well as through the secretion of soluble factors such as IL-10 and TGF-β (Shevach 2002).

iii. *Trl cells ($CD4^+FoxP3^-$):* These cells do not express $FoxP3^+$ and are induced into becoming Treg cells through a process of MHC/peptide stimulation in the presence of a suppressive cytokine, namely IL-10 (Groux et al. 1997, Grazia et al. 2006).

A number of additional peripherally-derived Treg cells have been identified including, the Th3 ($CD4^+TGF\text{-}\beta^+$) Treg cell population which, like Trl cells, has been shown to suppress cells through the secretion of IL-10 and TGF-β1 (Weiner 2001). These cells have been shown to inhibit the development of autoimmune diseases such as autoimmune encephalitis (Bridoux et al. 1997).

Recently a new population of suppressive $Th17FoxP3^+$ Treg cells have been identified in humans, with authors speculating that they may be essential in the regulation of

autoimmunity, inflammation and protection against bacterial pathogens (Voo et al. 2009). Th17FoxP3$^+$Treg cells are induced peripherally through the stimulation of the CD4$^+$FoxP3$^+$CCR6$^-$ Treg cell's T-cell receptor in the presence of human serum, IL-1β, IL-2, IL-21, and IL-23 (Voo et al. 2009). This results in the differentiation of these non-IL-17 secreting regulatory T-cells into IL-17 producing Treg cells (Voo et al. 2009). Numerous cytokines in combination with TGF-β have been shown to determine whether these cells become Treg suppressor cells, IL-17 producing T-helper cells or a combination of the two with both suppressor and effector function (Zhou et al. 2008).

1.3.2.2 CD8$^+$ Treg cells

CD8$^+$ Treg cells have also been identified following the demonstration of a population of Treg cells which did not conform to the CD4$^+$CD25$^+$FoxP3$^+$ phenotype (Kiniwa et al. 2007). Like CD4$^+$ Treg cells, these cells can also be thymically derived or peripherally induced (Kiniwa et al. 2007). In 2003, Cosmi and colleagues (2003) were the first to identify thymically derived CD8$^+$CD25$^+$GITR$^+$FoxP3 in the thymic tissue of humans, which have similar localisation, phenotypic function and mechanism of action as CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (Cosmi et al. 2003). Following activation, these CD8$^+$ Treg cells express TGF-β1 and CTLA-4 surface markers, which mediate the suppression of effector T-cell proliferation and activation through a cell-to-cell contact dependent mechanism (Cosmi et al. 2003). This interaction results in the suppression of IL-2Rα chain on effector T-cells, which renders them unresponsive to IL-2 (Cosmi et al. 2003). The absence of perforin or granzyme in CD8$^+$ Treg cells excluded the possibility of suppression being induced via apoptosis of the target effector T-cell (Cosmi et al. 2003). It has been suggested that these cells

play a crucial role in the regulation of T-cell mediated response against virally infected cells and malignant cells (Cosmi et al. 2003). Thereafter, Jarvis and colleagues (2005) also identified a population of $CD8^+CD25^+CTLA4^+FoxP3^+$ Treg cells in the peripheral blood of patients suffering from autoimmune Ankylosing Spondylitis. These cells were very similar to those found by Cosmi and colleagues (2003), except that they were shown to produce IL-4, IL-5 and IL-13, which was not demonstrated in the $CD8^+$ Treg population identified by Cosmi and colleagues (2003). These cells were also found to express CD69 continuously, which has been shown to induce the production of TGF-β1 by NK cells and T-lymphocytes (Jarvis et al. 2005). It is hypothesised that the $CD8^+$ Treg cell population identified within the thymus by Cosmi and colleagues (2003), may be precursors to the $CD8^+$ Treg cell population identified by Jarvis and colleagues in the peripheral blood circulation (Jarvis et al. 2005). This however is yet to be confirmed.

Numerous subpopulations of peripherally derived $CD8^+$ Treg cells have been identified, including: $CD8^+CD122^+$ Treg cell, $IL\text{-}10^+CCR7^+CD45RA^+CD8^+$ Treg cell and $CD8^+LAG\text{-}3^+CCL4^+$ Treg cell (Joosten et al. 2007, Kiniwa et al. 2007). These cells may be induced to become suppressive by immature dendritic cells, plasmacytoid dendritic cells, as well as malignant cells (Kiniwa et al. 2007). Following the finding of $CD8^+LAG\text{-}3^+CCL4^+$ Treg cells in granulomatous lesions of *Mycobaterium*-infected lymph nodes, it has been suggested that they may play a role in the regulation and promotion of chronic infection (Joosten et al. 2007).

$CD8^+$ Treg cells have been shown to inhibit the proliferation of antigen specific $CD4^+$ and $CD8^+$ effector T-cells through a cell-cell contact dependent mechanism, as well as

through the secretion of immunosuppressive soluble substances (Endharti et al. 2005, Kiniwa et al. 2007). Recently, the $CD8^+$ Treg cell population specific for heme-oxygenase-1 (HO-1-$CD8^+$) was identified in the peripheral blood of cancer patients (Andersen et al 2009). These cells are said to inhibit proliferation, cytotoxicity and cytokine production of antitumor effector cells including $CD4^+$ T-lymphocyte, $CD8^+$ T-lymphocyte and possibly NK cells (Andersen et al. 2009). Despite the identification of a variety of $CD8^+$ Treg subpopulations, there is still very little known about $CD8^+$ Treg cells in comparison to $CD4^+$ Treg cells which have been extensively researched. However, dysfunction of these cells have been implicated as major role players in the pathogenesis of cancer and autoimmune disease, thus opening up new avenues to be explored in the search for therapeutic interventions (Qiao et al. 2007, Andersen et al. 2009).

1.3.3 T regulatory cell function

Within healthy individuals, Treg cells have been shown to maintain immune tolerance by suppressing host immune response directed towards self-antigens, thus playing a crucial role in the prevention of the development of autoimmune diseases (Wilke et al. 2010). They contribute to the maintenance of tolerance to allogeneic tissues in transplantation medicine, with new studies demonstrating their ability to prevent both acute and chronic rejection of allografts (Pasquet et al. 2013). Furthermore, Treg cells limit tissue damage during inflammatory responses (D'Alessio et al. 2009), and play a significant role in the maintenance of fetomaternal tolerance during pregnancy (Habicht et al. 2007).

Treg cells have been shown to suppress host effector T-cell activity in numerous disease states and down-regulates T-cell function in antimicrobial (Lundgren et al. 2005), antiviral (Weiss et al. 2004) and antitumor response (Curiel et al. 2004, Taams et al. 2006). Over the last decade a substantial amount of attention has been given to the ability of Treg cells to down-regulate effector cells involved in mediating anticancer immunity, including NK cells (Trzonkowski et al. 2004), $CD4^+$ T-lymphocytes (Itoh et al. 1999), $CD8^+$ T-lymphocytes (Trzonkowski et al. 2004), NKT cells (Azuma et al. 2003), dendritic cells (DiPaolo et al. 2007) and B-lymphocytes (Lim et al. 2005).

Treg cells have been shown to mediate their immune suppressive mechanism in numerous overlapping ways, which may occur via a cell-to-cell directed mechanism or through the secretion of immunosuppressive substance.

These mechanism include:

i. Cytokine inhibition via release of IL-10, IL-35 and TGF-β (Grazia et al. 2006, Coombes et al. 2007, Collison et al. 2007);
ii. Direct cytolysis of effector T-cells by means of granzyme and perforin secretion (Gondek et al. 2005, Zhao et al. 2006);
iii. Metabolic disruption through the release of intracellular or extracellular adenosine nucleosides (Deaglio et al. 2007);
iv. Inhibition of dendritic cell function through binding of CTLA-4 to CD80/86 and induction of indoleamine 2,3-dioxygenase (IDO) (DiPaolo et al. 2007, Oderup et al. 2006);
v. Expression of negative costimulatory molecules (Joosten et al. 2007);
vi. Induction of anti-inflammatory biochemical pathways in effector T-cells and APCs (Andersen et al. 2009);
vii. Consumption of proinflammatory cytokines e.g. IL-2 (Pandiyan et al. 2007).

1.3.4 T regulatory cells and cancer

During the past two decades there has been a growing interest in the role that Treg cells play in the development and progression of cancer in vivo. This interest was triggered by the demonstration of increased levels of Treg cells in the peripheral blood of patients suffering from cancer, as compared to healthy individuals (Woo et al. 2001, Liyanage et al. 2002, Sasada et al. 2003, Liu et al. 2008, Jaberipour et al. 2010). Numerous studies have emerged which demonstrated an increase in the frequency of Treg cell activity in patients suffering from various cancers including solid tumors: lung (Petersen et al. 2006), pancreatic (Hiraoka et al. 2006), breast (Bates et al. 2006), liver (Ormandy et al. 2005), ovarian (Curiel et al. 2004) , gastric (Kono, a et al. 2006), oesophageal (Kono et al. 2006), cervical (Nakamura et al. 2007), head and neck cancer (Strauss et al. 2007) and haematological malignancies (Karube et al. 2004). This increase in Treg cells was identified in either the peripheral blood, regional lymph nodes or within the tumor microenvironment itself, with the highest numbers of Treg cells identified in patients with untreated or refractory disease (Kono et al. 2006, Sellitto et al. 2011).

This was followed by the investigation of Treg cells as a marker of prognosis, and numerous studies began to emerge which demonstrated an increase in the expansion of Treg cell populations in the peripheral blood of patients with metastatic disease (Nakamura et al. 2009, Wang, et al. 2012). The presence of Treg cells within the tumor microenvironment as well as the peripheral blood has been associated with a poor outcome in numerous cancers including ovarian, breast, gastric, oesophageal and non-small cell lung cancer (Curiel et al. 2004, Kono et al. 2006, Ohara et al. 2009, Xu et al. 2009, Merlo et al. 2009). Merlo and colleagues (2009) even went so far as to

suggest that the expression of FoxP3 in Treg cells correlates with the metastatic capability of cancer, instead of tumor immune suppression.

The general consensus in the medical world was that an increase in Treg cells correlated with poor survival, which was an exciting discovery as it would mean that a reliable prognostic marker for malignancy had been discovered. However, studies emerged which contradicted these findings and suggested that the presence of Treg cells within the tumor micro-environment correlated with improved patient outcome, and could even be used as an independent prognostic marker for better regional control of the malignancy (Alvaro, et al. 2005, Salama et al. 2009, Haas et al. 2009, Correale et al. 2010).

In clinical medicine there has been increased interest in possible methods of manipulating Treg levels in oncology patients, with the aim of improving response to antitumor treatment. This interest developed following observations that Treg cells impaired the efficacy of immunotherapeutic agents, and that the disruption of Treg cell pathways could result in the augmentation of immune surveillance or the facilitation of an immunotherapeutic cure (Nagai et al. 2004, Dannull et al. 2005). Multiple attempts have been made to deplete or block Treg cells in patients presenting with cancer and has resulted in the improvement of effector $CD8^+$ T-cell mediated antitumor response (Onizuka et al. 1999, Nagai et al. 2004, Dannull et al. 2005). Ontak (Denileukin diftitox), a fusion protein of diphtheria-toxin and IL-2, has received FDA approval for use in the treatment of persistent or relapsed $CD25^+$ cutaneous T-cell lymphoma (Manoukian 2009). Following the administration of Ontak, patients presenting with ovarian or renal cancer display reduced numbers of

peripherally circulating Treg cells, and improved T-cell activation (Barnett et al. 2005, Dannull et al. 2005). Additional pharmacological interventions directed at Treg cells have been proposed, including the use of agents directed against CTLA-4 (anti-CTLA-4-antibodies) e.g. Ipilimumab, in patients presenting with malignant melanoma (Hodi et al. 2003, O'Day et al. 2010), prostate cancer (Small et al. 2007) and ovarian cancer (Hodi et al. 2003). Following the administration of the anti-CTLA-4 agent, there was objective clinical improvement and improved long term survival, however the induction of autoimmune diseases in these patients following immunotherapy still remains a problem (Hodi et al. 2008, O'Day et al. 2010). Combination therapy (anti-CD25 and anti-CTLA-4), as well as anti-GITR antibodies are also being investigated and have given promising results; however, additional investigations need to be performed before these agents can be used in the clinical management of patients (Sutmuller et al. 2001, Ko et al. 2005).

1.4 Antitumor treatment and immunity

Surgery, chemotherapy and radiotherapy are the most commonly used antitumor treatment modalities in our setting. Although these anticancer therapies are effective in eradicating malignancies in a large proportion of cases, they have been shown to have a multitude of complications, including immunosuppression (Mafune et al. 2000). As more studies continue to assess the role of the host immune defence against cancer and possible ways in which new immunotherapeutic agents can facilitate these processes, conflicting research has emerged which question numerous dogmas about the more traditional antitumor interventions currently in use.

1.4.1 Surgical oncology

In an effort to evade effector T-lymphocytes and NK cells, malignant cells induce the Th1/Th2 balance to shift towards a Th2 response, thus inhibiting the induction of cell-mediated immunity and promoting a B-lymphocyte and humoral led immune response (Goto et al. 1999). This shift from a Th1 to a Th2 response has also been demonstrated in patients as a response to surgical stress (Ishikawa et al. 2009). Hesler and colleagues (1997) conducted a study where they assessed the immunological changes following major surgery. The indication for surgery was malignancy-related in 83% of cases; however, contrary to previous findings they did not observe any malignancy-induced immune dysregulation pre-operatively (Hensler et al. 1997). They did however demonstrate that surgery impaired the activation, proliferation and cytokine production of T-lymphocytes in the early post-operative period (day1-2) (Hensler et al. 1997) . Although Th1 cytokines and Th2 cytokines were impaired, the secretion of IL-10, an anti-inflammatory cytokine, was not impaired and continued to rise even in the late post-operative period (day3-5) (Hensler et al. 1997) . The levels of IFN-γ and IL-2 were seen to return to normal pre-operative levels in the late post-operative period, (day 5) (Hensler et al. 1997).

Similar findings were demonstrated by Mafune and colleagues (2000) who observed a decrease in NK cell activity in the post-operative period, in conjunction with an increase in the $CD4^+$:$CD8^+$ T-cell ratio secondary to diminished $CD8^+$ T-lymphocytes quantities. Prolonged immunosuppression following surgical intervention in cancer patients has been demonstrated in numerous studies (Yoshihara et al. 1986, Pollock et al. 1991, Hensler, et al. 1997, Ogawa et al. 2000), which may accelerate the development of pre-existing micrometastases and facilitate post-operative

dissemination of cancer and tumor metastases (Ben-Eliyahu et al. 1999, Mafune 2000, Goldfarb et al. 2011).

Despite the negative reports regarding surgical intervention in malignant conditions, proponents of surgical intervention claim that surgical excision of the tumor facilitates the immune response by decreasing the tumor cell burden, thus diminishing tumor associated mechanisms of antitumor immunity and decreasing the number of local Treg cells (González et al. 2007, Baumgartner et al. 2009). This results in the improvement of immune surveillance mechanism and possible impediment of tumor growth (González et al. 2007, Baumgartner et al. 2009). Studies have demonstrated improved survival following surgical excision of the primary tumor mass in patients presenting with renal cell carcinoma (Flanigan et al. 2001), colorectal (Rosen et al. 2000), breast (Gnerlich et al. 2007) and ovarian cancer (Griffiths et al. 1978). Leung and colleagues (2010) conducted similar studies in stage IV breast cancer patients and found that patients who received surgical treatment had a survival benefit over patients who received no surgical treatment. This finding corresponded with previous findings (Leung et al. 2010). However, following the standardisation of results for patients who received chemotherapy in addition to surgical treatment, they concluded that surgical intervention in itself does not provide a survival benefit but the addition of a chemotherapeutic regimen does (Leung et al. 2010).

1.4.2 Chemotherapy

Chemotherapy is the process whereby chemical agents with cytotoxic activity are administered systemically to patients with the aim of eradicating or controlling the cancer growth (Morgan 2003). The use of chemotherapeutic agents in clinical

medicine has spanned numerous decades, and has been shown to be beneficial in a multitude of conditions including autoimmune diseases, transplantation medicine and lymphoproliferative conditions (Rasmussen et al 1982). Chemotherapeutic agents however target rapidly replicating cells in a non-selective manner thus malignant cells as well as normal cells are targeted (Rasmussen et al 1982); consequently, the majority of oncology patients receiving chemotherapy experience chemotherapy-induced immune dysregulation (Lehrnbecher et al. 2008, Chu-Yuan et al. 2013). Chemotherapy has been shown to affect multiple components of the immune system starting with the innate immune defence where chemotherapy-induced mucositis breaks down the external protective barrier (Ek et al. 2005, Fanning et al. 2006). NK cells are shown to decline significantly during treatment and although NK cell levels recover following treatment, studies have suggested that due to their crucial role in antitumor defence, such a significant decline may contribute to cancer relapse (Lehrnbecher et al. 2008). The functional capacity, chemotactic ability and frequency of neutrophils have also been shown to be affected with patients presenting with a clinical condition known as febrile neutropenia, which consists of a fever of >38°C and an total neutrophil count of <1.0 x 10^9 cells/l (Lehrnbecher et al. 1997, Mendonça et al. 2006, Naik et al. 2010). This condition renders the patient susceptible to life threatening bacterial infection (Naik et al. 2010).

Numerous studies have demonstrated the severe chemotherapy-induced lymphopenia associated with the majority of chemotherapeutic agents, with B-cell, $CD4^+$ and $CD8^+$ T-cell populations being affected (Sewell et al. 1993). The remaining circulating lymphocytes have also been shown to have impaired effector function thus rendering the patient susceptible to opportunistic infections (Lehrnbecher et al. 2008). Follow

up of these patients have also demonstrated that the frequency of circulating $CD4^+$ T-lymphocytes remains low for up to six months following treatment; however, the frequency of $CD8^+$ T-lymphocyte is said to recover one month after chemotherapy (Ek et al. 2005).

One of the benefits of the global lymphopenia induced by the chemotherapy is the fact that no T-lymphocyte population is spared, including Treg cells. Treg cells have been shown to be extremely sensitive to the chemotherapeutic agent cyclophosphamide (Ghiringhelli, Larmonier et al. 2004, Traverso, Fenoglio et al. 2012). Cyclophosphamide, a nitrogen mustard alkylating agent, is one of the most commonly used chemotherapeutic agents both in the treatment of cancer and autoimmune disease (Emadi et al. 2009, Brodsky et al. 2010). It has been shown to inhibit $CD4^+$ and $CD8^+$ Treg cell proliferation and suppressive activity, with Treg proponents suggesting that it should be regarded as a vital component of any antitumor treatment protocol (Lutsiak et al. 2005, Traverso et al. 2012).

Following this finding, it was assumed that alternative chemotherapeutic agents could also inhibit Treg cell proliferation and activity. However, Tohyama and colleagues (2013) conducted a study which assessed the influence of 4 widely used chemotherapeutic agents (methotrexate, arsenuc trioxide, dacarbazine and 5-fluorouracil) on the cellular component of the immune system of healthy human participants. They found that not only did these agents induce a state of neutropenia, but they also decreased the quantity of effector T-cells and inhibited effector T-cell proliferation and function (Tohyama et al. 2013). Interestingly, they were shown to increase the relative numbers of Treg cells, possibly through the elimination of other

T-cell subsets (Tohyama et al. 2013). Thus raising many questions regarding the possibility of Treg cells being chemo-resistant, as well as the possibility that certain chemotherapeutic agents attenuate the antitumor immune response, and tilt the balance towards a more immunosuppressive tumor-microenvironment, which augments tumor growth and progression.

1.4.3 Radiotherapy

The use of external beam radiotherapy in the treatment of cancer is also extremely common and can be used as a primary form of treatment (De-en, et al. 1988), or in combination with additional antitumor treatments, such as chemotherapy or surgery (Standish et al. 2008, Kang et al. 2009, Kachikwu et al. 2011). As already mentioned, surgery and chemotherapy suppress the patients immune response, and the use of radiotherapy as a supplementary antitumor intervention in these patients enhances the level of immune suppression even further (Standish et al. 2008, Standish et al. 2008b).

Standish and colleagues (2008b) conducted a study which assessed the influence of external beam radiotherapy on the innate and adaptive immunity of patients being treated for breast cancer. Following a standard course of external beam therapy, patients presented with a significant decline in the lymphocyte population which did not return to normal levels at 6 weeks after the termination of treatment (Standish et al. 2008b). This persistent lymphopenia was also observed in a study conducted by Bachtiary and colleagues (2005), where patients receiving radiation treatment for cervical cancer developed severe immunosuppression, which rendered them susceptible to opportunistic infections (Bachtiary et al. 2005). $CD8^+$ T-lymphocytes are more radiosensitive than $CD4^+$ T-lymphocytes, with studies showing an increase

in the $CD4^+$: $CD8^+$ T-cell ratio shortly after radiotherapy (Mafune et al. 2000). Radiotherapy has also been shown to reduce the frequency and effector activity of monocytes and NK cell, however neutrophils seem to be radio-resistant as they were not affected by the radiation and remained within normal range (Standish et al. 2008b).

Similar to neutrophils, studies have demonstrated that Treg cells do not decline following exposure to radiotherapy (Lissoni et al. 2009, Kachikwu et al. 2011). Some have even suggested that they may be the most radio-resistant T-cell subset following findings by Lissoni and colleagues (2009) who demonstrated a significant decline in the total lymphocyte count and $CD4^+$ T-cell count, while no change was observed in the circulating Treg cell count in cancer patients who received a course of radiotherapy. Some studies have even demonstrated that Treg cells increase following exposure to radiotherapy (Horwitz et al. 2008, Kachikwu et al. 2011). This increase is said to be secondary to radiation induced secretion and activation of TGF-β, which has been shown to induce the peripheral conversion of $CD4^+CD25^-$ T-cells into Treg cells, thus exacerbating the state of immune suppression and promoting tumor tolerance (Horwitz et al. 2008, Kachikwu et al. 2011).

1.5 Breast cancer and immunology

1.5.1 Breast cancer epidemiology

Breast cancer (BCA) is the most common malignancy affecting women in sub-Saharan Africa, as well as the most common cause of cancer-related deaths in this region (Ferlay et al. 2010, American Cancer Society 2011). In the last 2 decades, sub-Saharan Africa has seen a significant rise in the prevalence of BCA (American Cancer Society 2011). This has been attributed to an increase in economic development, which is associated with increased BCA risk factors including obesity, early menarche, late child bearing and having fewer children (American Cancer Society 2011). It may also be attributed to improvements in access to healthcare facilities, which would increase the identification of patients suffering from BCA, as well as improved screening methods, increased self examination and increased BCA community awareness programs which would allow for the identification of malignancies which are not yet clinically detectable (Kingham, et al. 2013).

According to the World Bank, South Africa is classified as a middle-income country and has a diverse population consisting of 52 982 000 people (Stats SA 2013, World Bank 2014). Despite the fact that the only available national cancer statistics for South Africa dates back to 2006, the statistics are still similar to what is currently being observed in the rest of sub-Saharan Africa, with BCA still being the most common cancer in women in South Africa (Figure 1.7). The current age standardised incidence rate of BCA in South Africa is 29.29/100 000, and a South African woman's lifetime risk of developing BCA is 1 in 33 (National Health Laboratory Services 2014, CANSA 2014, African Cancer Registry Network 2014).

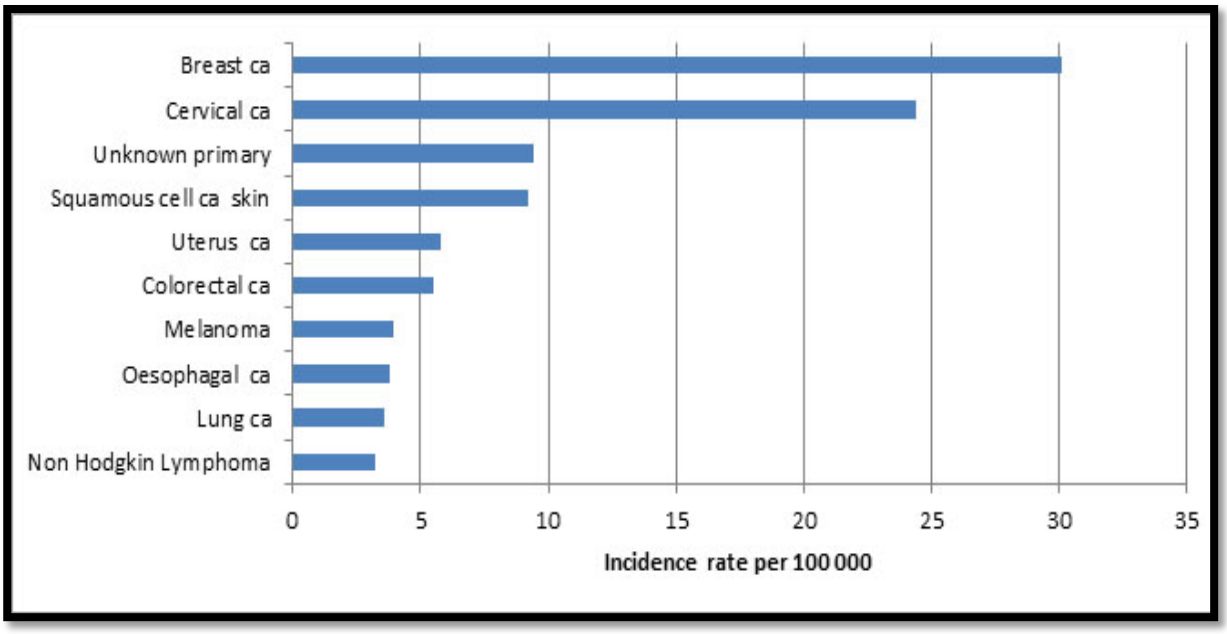


Figure 1.7: Incidence of cancer in South Africa (African Cancer Registry Network 2014)

South Africa has been shown to have a higher BCA mortality: incidence ratio as compared to the world standard, which suggests that patients suffering from the condition in South Africa have a higher risk of death (Wadler et al. 2011). This is in agreement with reports stating that women in developing countries who are diagnosed with BCA have a poorer prognosis then those in developed countries (Ferlay et al. 2010). Despite all efforts to keep track of the evolution of BCA in South Africa through the National registry, there is a tendency to underestimate the true incidence of cancer as these statistics only represent pathology-based incidences rather then reporting a population-based incidence of BCA (Vorobiof et al. 2001, Wadler et al. 2011).

1.5.2 Pathology

BCA is regarded as an epithelial malignancy which affects the breast tissue and is classified according to the cytolytic features, biological behaviour and growth patterns of the malignancy. There are two main groups of BCA, including Ductal (intraductal) carcinoma insitu and lobular carcinoma insitu, which are further subdivided into several histological subtypes including; infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary, papillary, metaplastic and invasive micropapillary breast cancer (Schnitt et al. 2004, Li et al. 2005). Infiltrating (invasive) ductal carcinoma insitu is the most commonly occurring histological type of breast cancer, accounting for 50-80% of diagnosed BCA (Table 1.2) (Weigelt et al. 2005).

1.5.3 Breast cancer prognosis

The search for a molecular marker that can be used as a prognostic and predictive marker in BCA is still on going with minimal success (Perez et al. 2007). Numerous potential markers have been identified (Table 1.3)(Weigelt et al 2005); however, their value in the treatment of BCA is continuously being questioned because of the lack of adequate quality control and the absence of the validation of these markers in an adequately powered clinical trial (Esteva et al. 2004). Despite the deficiencies of some of these markers, they are still being used during the assessment and management of BCA

Histopathological type of invasive breast carcinoma	Frequency	10-year survival rate
Invasive ductal carcinoma, not otherwise specified	50–80%	35–50%
Invasive lobular carcinoma	5–15%	35–50%
Mixed type, lobular and ductal features	4–5%	35–50%
Tubular/invasive cribriform carcinoma	1–6%	90–100%
Mucinous carcinoma	<5%	80–100%
Medullary carcinoma	1–7%	50–90%
Invasive papillary carcinoma	<1–2%	Unknown
Invasive micropapillary carcinoma	<3%	Unknown
Metaplastic carcinoma	<5%	Unknown
Adenoid cystic carcinoma	0.1%	Unknown
Invasive aprocrine carcinoma	0.3–4%	Unknown
Neuroendocrine carcinoma	2–5%	Unknown
Secretory carcinoma	0.01–0.15%	Unknown
Lipid-rich carcinoma	<1–6%	Unknown
Acinic-cell carcinoma	7 cases	Unknown
Glycogen-rich, clear-cell carcinoma	1–3%	Unknown
Sebaceaous carcinoma	4 cases	Unknown

Table 1.2: Histopathological types of invasive breast cancer (Weigelt et al. 2005)

.

The most commonly used markers of prognosis in our setting includes:

i. *Tumor size and axillary node status:* Both tumor size and nodal status has been shown to have independent adverse effects on prognosis (Carter et al. 1989, Gupta et al. 2007). The use of tumor size to determine prognostic status has been used for more than 4 decades with studies demonstrating low risk of metastasis in patients presenting with tumor size <2cm and patients having a high risk of metastasis when presenting with tumor size >5cm (Carter et al. 1989, Weigelt et al. 2005). Lymph node involvement is also commonly assessed and is always considered in conjunction with the tumor size, with the effect of size on prognosis being greater in patients presenting with no nodal involvement (Carter et al. 1989). Irrespective of tumor size, patients presenting with the involvement of adjacent lymph nodes have a worse outcome then node negative patients, and if found to have tumor invasion of ≥4 axillary lymph nodes they have a significantly poorer outcome and the highest risk of metastasis (Carter et al. 1989, Weigelt et al. 2005).

Marker	Use in clinic	Metastatic determinants	Details
Tumour size	Established	Tumours under 2 cm in diameter have a low risk of metastasis; tumours of 2–5 cm have a high risk of metastasis; tumours over 5 cm have a very high risk of metastasis	Independent prognosis marker
Axillary lymph-node status	Established	If there are no lymph-node metastases, the risk of metastasis is low; if lymph-node metastases are present, the risk of metastasis is high; the presence of over 4 lymph-node metastases is associated with very high metastasis risk	Related to tumour size
Histological grade	Established	Grade 1 tumours have a low risk of metastasis; grade 2 tumours have an intermediate risk of metastasis; grade 3 tumours have a high risk of metastasis	Related to tumour size
Angioinvasion	Established in patients with lymph-node-negative tumours	The presence of tumour emboli in over 3 blood vessels is associated with metastasis	In patients with lymph-node-negative tumours
uPA/PAI1 protein level	Newly established marker	High protein levels of uPA and PAI1 are associated with high metastasis risk	Independent prognosis marker
Steroid-receptor expression	Established for adjuvant therapy decision	Low steroid-receptor levels are associated with metastasis	Short-term predictor of metastasis risk (5 years); related to histological grade
ERBB2 gene amplification and protein expression	Established for adjuvant therapy decision	*ERBB2* amplification/overexpression is associated with metastasis	In patients with lymph-node-positive tumours
Gene-expression profiling	Currently being tested	A 'good signature' of 70 genes is associated with low metastasis risk; a 'poor signature' of 70 genes is associated with high metastasis risk	Tested in patients with lymph-node-negative tumours

PAI1, plasminogen activator inhibitor 1; uPA, urokinase-type plasminogen activator.

Table 1.3: Breast cancer metastatic determinants (Weigelt et al. 2005)

ii. *Histological grade:* In 1991 Elston and colleagues (1991) graded BCA according to 3 categories based on the architectural and cytological features, which include: well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3) BCA. They demonstrated that patients whose malignancy was classified as a grade 3 tumor (poorly differentiated) had a significantly worse prognosis then those presenting with grade 1 or 2 tumors (Elston et al. 1991).

iii. *Human Epidermal growth factor 2 receptors (HER-2/neu)*: This proto-oncogene, which is also known as c-*erb*B-2 or *neu*, is over-expressed in 60% of ductal carcinomas insitu and 20-30% of infiltrating breast carcinoma (Slamon et al. 1987, Esteva et al. 2004). It is the most commonly used prognostic and predictive marker in breast cancer and the assessment of HER-2/neu status is regarded as the standard of care in the management of BCA (Esteva et al. 2004, Weigelt et al. 2005). Over-expression of HER-2/neu receptors in patients with breast cancer, are associated with a poor prognosis and shortened disease free survival rate in patients with axillary node positive BCA (Allred et al. 1992). The presence of the HER-2/neu receptor has also been shown to dictate the treatment protocol, following an observed beneficial response to Trastuzumab (a monoclonal antibody directed against HER-2/neu), hormonal therapy and doxorubicin-based chemotherapy in patients with increased expression of the receptor (Paik et al. 1998, Slamon et al. 2001). However, over-expression of

HER-2/neu has also been shown to confer radio-resistance and is associated with ipsilateral breast cancer recurrence following radiotherapy (Akamatsu et al. 2003). Soluble forms of HER-2/neu receptor (HER-ECD) is currently being evaluated as a predictive marker of treatment response, however there are still a number of limitations which need to be addressed before it can be used in the clinical setting (Kostler et al. 2004, Kostler et al. 2004b, Perez et al. 2007).

iv. *Estrogen and progesterone receptor status*: The over-expression of estrogen receptors (ER-α) in BCA is a well-known positive prognostic marker, and facilitates the identification of patients who will benefit from hormonal treatment (Esteva et al. 2004). The incidence of BCA in African women, as compared to Caucasian women, is relatively low; however, only a small proportion of African women who develop BCA express ER positivity. Furthermore, a higher proportion of African women over-express the HER-2/neu receptor, which may contribute to the poorer outcomes observed in Africa women (Bird et al. 2008, Bogaert 2013). The contribution of progesterone receptor status in the prognostication of BCA is purely as a functional assay to ensure that the estrogen pathway is intact (Esteva et al. 2004). Although ER status is commonly used and facilitates treatment, the assays used to determine estrogen status are not standardized and the definition of positivity differs between laboratories (Esteva et al. 2004).

v. Proliferation markers: Ki-67 is a nuclear antigen expressed during the proliferative stages of the cell cycle and has been shown to be over-expressed in

breast cancer patients with increased risk of recurrent disease (Veronese et al. 1993).

Despite the large number of prognostic markers currently available in clinical use, the deficiencies associated with traditional prognostic markers result in the identification of only 30% of patients with either the best and worse prognosis (Weigelt et al. 2005).

1.5.4 Breast cancer and T regulatory cells

Following the discovery of Treg cells, their abundance within the tumor microenvironment of a variety of cancers, and their possible contribution to tumor progression, numerous studies began to investigate the role of Treg cells in BCA. The first study to demonstrate the presence of Treg cells in breast cancer was by Liyanage and colleagues (2002), who showed that the frequency of Treg cells within the peripheral blood and tumor microenvironment of patients with BCA was significantly higher than that in healthy controls. They also found no difference between the prevalence of Treg levels in healthy controls and patients who presented with benign disease (Liyanage et al. 2002). Liyanage and colleagues (2002) did not assess FoxP3 expression within the Treg cell population, as suggested by the literature, with only CD4, CD25, CTLA-4 and CD152 being used as markers of the suppressive Treg cell population; however, suppressive activity was confirmed with the use of functional assays. These findings were replicated in numerous studies thereafter (Bates et al. 2006, Ladoire et al. 2008, Ohara et al. 2009) and gave strong evidence to suggest that Treg cells have a crucial role in the development and progression of breast cancer.

Questions regarding the use of Treg cells as a prognostic factor began to arise and Bates and colleagues (2006) were the first to show that elevated intratumoral

FoxP3^{+}Treg cells correlated significantly with a more aggressive tumor phenotype, higher tumor grade and positive nodal status. They identified a novel group of high-risk patients whose tumors expressed ER-positivity, but had as poor a prognosis as patients with ER-negative tumors, with high intratumoral Treg levels being the only identifying feature (Bates et al. 2006). They observed that patients with elevated intratumoral FoxP3^{+}Treg cells had a shorter overall survival (Hazard Ratio (HR) 1.85 and relapse-free survival (HR 1.98 (Bates et al. 2006). The frequency of FoxP3^{+}Treg cells within the tumor was deemed as a marker of prognosis in invasive and non-invasive BCA and a marker for identification of patients with a risk of late relapse (Bates et al. 2006). These findings were also replicated in other studies (Ladoire et al. 2008, Aruga et al. 2009), including a study by Ladoire and colleagues (2008), who observed that elevated levels of intratumoral CD8^{+} T-cells was also associated with poor prognosis.

The functional status of FoxP3 expression in breast cancer has also yielded interesting results, with increased expression of FoxP3 being associated with more invasive breast carcinomas, as compared to carcinoma insitu or normal tissue (Gupta et al. 2007). Gupta and colleagues (2007) demonstrated higher intratumoral FoxP3 expression in patients who presented with stage III BCA, as compared to patients presenting with stage II BCA. They also found a linear association between FoxP3 expression and tumor size, and suggest that FoxP3 expression is a marker of tumor progression and metastasis in breast carcinoma (Gupta et al. 2007).

The frequency of FoxP3^{+}Treg cells within the peripheral blood has also been shown to correlate with the stage of disease, with patients demonstrating significantly higher

levels of Treg cells in patients presenting with metastatic disease, as compared to those presenting with either stage I, II or III BCA (Rech, et al. 2010). The metastasis of BCA from the primary cancer site to other sites occurs via haematogenous, lymphatic and direct routes, with preferential sites including the lungs, brain, liver and bones (Tannock et al. 1998, Nguyen et al. 2007). Invasion into the adjacent axillary lymph nodes continues to be one of the most important determinants of cancer progression, especially when this involves the sentinel lymph nodes, which are located on the lymphatic drainage pathway of the breast (Watanabe et al. 2010). The accumulation of $FoxP3^+$ Treg cells within these Sentinel lymph nodes have also been shown to be associated with undetected micrometastases, and is an independent predictor of prognosis in patients presenting with node negative cancer (Nakamura et al. 2009).

The Treg cell population has also been suggested as a predictive marker of treatment response following a study by Ladoire and colleagues (2008) who observed a significant decrease in Treg cell frequency in BCA following complete histological response to neo-adjuvant chemotherapy, independent of chemotherapeutic regimen, compared to non responders. However, cyclophosphamide was included as part of the chemotherapeutic regimen, which may be responsible for the observed responses, as previously discussed (Ladoire et al. 2008).

The presence and activity of Treg cells in BCA patients has also been shown to correlate with the more traditional prognostic and predictive markers (Perez et al. 2007). Perez and colleagues (2007) demonstrated a significantly higher level of peripherally circulating Treg cells in patients with HER^+ breast cancer compared to

HER$^-$ breast cancer, and normal controls. Ohara and colleagues (2009) also observed elevated levels of circulating Treg cell in patients presenting with HER2$^+$ breast cancer, as well as in patients presenting with progesterone receptor negative BCA. Although Horlock and colleagues (2009) also observed similar results, unlike Perez and colleagues, they found that patients presenting with HER$^+$ and HER$^-$ breast cancer had similar levels of Treg cell.

Perez and colleagues (2007) observed a significant decrease in Treg cell frequency in patients who showed a good clinical response to Trastuzumab therapy. Furthermore, one month before the clinical confirmation of disease progression in patients with disease recurrence, Treg levels were shown to increase significantly (Perez et al. 2007). This was confirmed in a study by Horlock and colleagues (2009), who observed a significant decrease in patients presenting with metastatic BCA following treatment with Trastuzumab. Perez and colleagues (2007) proposed the use of circulating Treg levels as a surrogate marker of response to Trastuzumab therapy in patients with BCA.

Although it is logical to assume that Treg cell possibly plays a role as a prognostic marker in breast cancer, there are still numerous conflicting reports and unanswered questions. Further studies need to be conducted assessing the relationship between Treg cells in breast cancer and how it can be manipulated in the management of patients presenting with BCA.

1.6 Oesophageal cancer and immunology

1.6.1 Oesophageal cancer epidemiology

Oesophageal cancer is the 8th most common cancer in the world and is the only cancer which has shown a steady increase in incidence across the world (Tettey et al. 2013, World Cancer Research Fund International 2013). According to the 2008 International Agency for Research on Cancer (IARC) statistics, South Africa has the highest incidence of oesophageal cancer in the world, and is the 3rd most common cause of cancer both in men (incidence rate: 23.5/100000) and women (incidence rate 12.6/100000) (IARC 2013).

Oesophageal cancer is associated with a significant morbidity and mortality and despite advances in multimodal anticancer treatment, the five year survival rate of these patients' remains below 10% (Pickens et al. 2003). This has been attributed to the late stage of presentation and consequent advanced stage of disease, which results in patients only being eligible for palliative treatment (Liakos et al. 2010, Tettey et al. 2013).

1.6.2 Pathology

Eighty percent of primary oesophageal cancers are malignant in nature with 98% of histopathological types being squamous cell carcinoma (SCC) and adenocarcinoma (AdenoCa) (Patel et al. 2003, Pickens et al. 2003). The remaining cancer types include sarcoma (1%), lymphoma (0.5%), cylindroma (0.2%) and primary melanoma

(0.25%) (Patel et al. 2003). Despite reports demonstrating a decline in the incidence of SCC, it is still the most commonly occurring histological type of oesophageal cancer worldwide, and has been found to be 5 times more common in African patients as compared to Caucasian patients (Brown et al. 2001, Pickens et al. 2003, Tettey et al. 2013). The increased risk of Africans to develop SCC has been attributed to a possible genetic predisposition, as well as the exposure to certain risk factors including; low-income status, increased alcohol consumption, heavy smoking and low intake of raw fruit and vegetables (Brown et al. 2001, Pickens et al. 2003). The incidence of adenocarcinoma is showing an upward trend and is associated with a premalignant condition called Barret metaplasia, which develops following a prolonged duration and severity of gastro-oesophageal reflux disease (Patel et al. 2003).

1.6.3 Oesophageal cancer prognosis

The mortality rate associated with oesophagus cancer is almost 100% (Patel et al. 2003), and the prognosis of oesophageal cancer remains poor despite aggressive multimodal treatment (Liakos et al. 2010) . Early detection of malignancies offers the best prognosis, however very few prognostic and predictive factors have been identified for clinical use in oesophageal cancer (Wang et al. 2011). There have been numerous studies looking at the possible role of Ki-67, p53, *HER-1* and ProExC expression in detecting early oesophageal cancer and serving as a prognostic and predictive marker. Yang and colleagues (1999) observed that p53 decreases the responsiveness of the malignancy to chemoradiotherapy, however these observations were not found in other studies (Akamatsu et al. 2003). Ki-67 and ProExC are both markers of cell proliferation and have been shown to identify early stage oesophageal

cancer (Wen-Chuang et al 2011). As in breast cancer, the over expression of *HER-1* or *HER-2* receptors are associated with a significantly worse outcome (Vallböhmer et al. 2006). Furthermore, a study by Akamatu and colleagues (2003) found that *HER-2* over-expression correlated with a poor prognosis, rendered the cells radio-resistant and induced local resistance to cisplatinum-based chemoradiotherapy (Akamatsu, Matsumoto et al. 2003).

Traditional prognostic factors have been assessed including tumor size, nodal status and metastatic spread, however they have been shown as insufficient for accurate prognostication of early recurrence in oesophageal cancer (Zhu et al. 2011). Zhu and colleagues (2011) identified additional prognostic factors; including grade of differentiation, depth of invasion, lymph node metastasis, number of lymph node metastases and margin status. These factors were shown to predict early death by recurrence of the primary tumor following the removal of the primary tumor (Zhu et al. 2011).

1.6.4 Oesophageal cancer and T regulatory cells

Oesophageal cancer patients are presumed to be in a poorer immunological state in comparison to other oncology patients due to the malnutrition associated with dysphagia (Mafune et al. 2000). Furthermore, aggressive multimodality treatment, such as major surgery with thoracotomy and laparotomy, combined radiotherapy and adjuvant chemotherapy further depress the immunological state (Mafune et al. 2000). Although the clinical and pathological features of oesophageal cancer at time of diagnosis have been established as prognostic factors, host defences against cancer are also thought to contribute to prognosis (Tsutsui et al. 1996). Despite increasing

clarity in the relationship between nutritional status, tumor staging and immunological parameters in patients with oesophageal cancer, the search for immunological markers of prognosis has not yielded any conclusive results (Mafune et al 2000). This is further complicated by the fact that there is controversy regarding whether the hosts immunological parameters can accurately be identified from the peripheral blood (Mafune et al. 2000). This has resulted in numerous studies focusing on the immunohistochemical analysis of post-oesophagectomy tumour specimens for prognostic and predictive parameters (Tsutsui et al. 1996, Schumacher et al. 2001, Xu et al. 2009, Xu et al. 2011).

Cho and colleagues (2003) investigated the prevalence of immune cells within oesophageal tumor samples and observed that patients presenting with high quantities of $CD4^+$ and $CD8^+$ T-lymphocytes within the stroma and cancer cell nest had a better prognosis compared to other groups. The balance between $CD4^+$ and $CD8^+$ T-lymphocytes has been suggested as a critical marker in the prognosis of patients with oesophageal cancer, furthermore $CD4^+$: $CD8^+$ T-cell status was suggested as a possible prognostic marker and index of post-surgical host immune reactivity (Cho et al. 2003) . This substantiated the use of $CD4^+$: $CD8^+$ T-cell ratio as a prognostic marker in immunohistochemical analysis, however the use of these cells as markers of prognostication when sampled from the peripheral blood still remains unanswered.

Yoskioka and colleagues (2008) conducted a similar study; however, in addition to $CD4^+$ and $CD8^+$ T-cells, they also assessed the frequency of Treg cells within the tumor microenvironment. They found that high quantities of intratumoral $FoxP3^+$Treg cells correlated with improved prognosis in patients presenting with early and

advanced oesophageal cancer (Yoshioka et al. 2008). Furthermore, Yoshioka and colleagues (2008) also observed that Treg cell status did not correlate with the TNM classification, and had no influence on the progression of the malignancy. Treg cell populations were not shown to have any suppressive effect on effector $CD4^+$ and $CD8^+$ T-cells, and elevated levels of Treg cells were associated with an increased $CD4^+$: $CD8^+$ T-cell status (Yoshioka et al. 2008). This contradicted all studies that claimed that high Treg cell levels correlated with poor prognosis, and it was suggested that the presence of Treg cells within the microenvironment is compatible with a normal anticancer immune response (Yoshioka et al. 2008).

Studies then started assessing whether similar results would be found in the peripheral blood of patients with oesophageal cancer. A study by Xu and colleagues (2009) demonstrated elevated levels of Treg cells in the peripheral blood of patients presenting with oesophageal cancer. Furthermore, Treg: $CD4^+$ T-cell ratio and $FoxP3^+$ expression was significantly decreased following chemotherapeutic treatment (Xu et al. 2009).

In summary breast and oesophageal carcinomas are extremely prevalent conditions in our setting, which are associated with significant morbidities and mortalities. The immune system has now been shown to have a substantial influence on the patients' health outcomes following the diagnosis of cancer. This has also led to the search for an immunological marker, which will serve as a prognostic, and predictive marker that facilitates the management of oncology patients. The discovery of Treg cells has rejuvenated the search for an explanation for immune escape; however, there are still numerous inconsistencies and conflicting findings regarding the T regulatory cell

population. Despite these discrepancies, if the Treg cell population proves to be a valid prognostic and predictive indicator in the management of cancer, it will guide treatment practices and facilitate the monitoring of patients receiving anticancer treatment. Furthermore, it will facilitate the identification of patients who are treatment resistance and enable the cessation of unnecessary therapy in patients with poor prognosis.

2 Aim and objectives

2.1 Aim

This study aimed to evaluate whether the levels of T-lymphocyte subsets, and alterations of these levels within the peripheral blood of HIV-negative patients presenting with either breast or oesophageal cancer at Steve Biko Academic hospital and Kalafong hospital, at the onset of treatment as well as following conventional anticancer treatment (surgery, chemotherapy and radiotherapy) could be used as markers of prognosis in conjunction with other well known prognostic factors, or as predictors of short term survival.

2.2 Objectives

2.2.1 Primary objectives

- To assess the relationship between levels of peripheral $CD4^+$, $CD8^+$ and Treg T-lymphocyte subsets and prognosis in patients presenting with breast cancer or oesophageal cancer.
- To assess the relationship between circulating levels of $CD4^+$, $CD8^+$ and Treg T-lymphocyte subsets and 7 weeks post-treatment survival of breast and oesophageal cancer patients.

2.2.2 Secondary objectives

- To assess the distribution of tumor stage at presentation in South African patients presenting with breast or oesophageal cancer.

- To quantify the T-lymphocyte immune profile in South African patients presenting with breast and oesophageal cancer.
- To assess the efficacy and agreement of the two different techniques used in the characterization and measurement of circulating Treg cell subsets.

3 Study cohort

3.1 Setting

Study participants were identified at the Surgery outpatient clinic at Kalafong Hospital and Steve Biko Academic Hospital, Pretoria, Gauteng, South Africa.

3.2 Eligibility criteria

Inclusion criteria:

1. Histological diagnosis of breast cancer or oesophageal cancer.
2. Human Immunodeficiency Virus negative status confirmed by serological test (ELISA testing). HIV-infection in the human population has been shown to promote T-cell activation and induce a progressive decline in $CD4^+$ and $CD8^+$ T-lymphocytes (Altfeld et al. 2000, Fiore et al. 2006). Furthermore, Treg cells have been shown to be elevated in HIV-positive individuals compared to healthy controls (Suchard et al. 2010). In light of the influence of HIV-infection on these variables of interest, the inclusion of HIV-infected participants into the study would serve as a substantial confounding factor, and would prevent the assertion of whether the observed effect was as a result of the antitumor treatment which the participant received or whether it was a result of the HIV-infection.

Exclusion criteria:

1. Refused to give consent to partake in the study.
2. Had received any antitumor or immunosuppressive treatment during the preceding 12 months as this would serve as a confounding factor.

3.3 Breast cancer cohort

All patients seen at the Family Medicine Clinic and Casualty Department with clinical symptoms suggestive of breast cancer where referred to the Surgery Outpatients Department, where a core needle biopsy of the breast mass was taken. Once a histological diagnosis of breast cancer was confirmed, participants were referred to the surgical-oncology team, consisting of surgeons, oncologists and radio-oncologists, for multi-modal treatment.

Staging of the malignancy was conducted following a number of investigations including: radiological examination, mammography, nuclear studies, electrolyte and haematological studies. Radical versus palliative treatment options were proposed for each patient after consideration of the following factors:

- TNM staging according to the American Joint Committee on Cancer (AJCC);
- Hormonal and HER-2/neu receptor status (ER, PR and HER);
- Comorbidities, especially those rendering the patient unable to tolerate anaesthetic;
- Performance status.

Depending on the factors specified above, participants received a combination of the following treatment interventions:

- Chemotherapy;
- Radiotherapy;
- Surgery (Mastectomy/ lumpectomy);
- Hormonal treatment (Tamoxifen).

3.4 Oesophageal cancer cohort

All patients seen at the Family Medicine and Casualty Department at Steve Biko Academic Hospital and Kalafong Hospital with signs and symptoms suggestive of oesophageal cancer were referred to the Surgery Outpatients Department. In this cohort it was noted that the majority of oesophageal cancer patients presented in a substantially poorer physical state compared to those suffering from breast cancer, with 7 out of the 10 participants presenting with symptoms of moderate to severe inability to swallow, dehydration, severe wasting and malnutrition.

Participants were admitted to the surgical wards for rehydration and oncology work-up which included a barium swallow for identification of the anatomical level at which the malignancy was situated, and a gastroscopy-guided biopsy and dilatation of the oesophageal mass. The surgical-oncology team proposed an individualised palliative or radical treatment plan depending on:

- TNM-staging of oesophageal carcinoma;
- Performance status;
- Length of the lesion and degree of narrowing;
- Associated comorbidities.

Treatment was then divided into:

- Radical treatment (chemotherapy and/or radiotherapy and, or surgery);
- Palliative treatment (Radiotherapy and/or surgical-bypass).

3.5 Recruitment

Between March 2012 to August 2012 participants were approached to volunteer for the current study following the clinical and histological confirmation of breast or oesophageal cancer. All potential participants were required to undergo an HIV (ELISA) test before admission into the study.

Each participant served as their own control and they were recruited into the study once they gave written informed consent. All research protocols were submitted to the University of Pretoria Ethics Committee, and the recruitment of participants began once final approval had been granted.

The breast cancer cohort comprised of 25 women (17 African and 8 Caucasian) with a mean age of 59, (range: 38-87). (Shown in Table 3.1) The oesophageal cancer cohort comprised of 10 participants (7 females and 3 males), with a mean age of 62 years (range: 41-85) amongst the females and 61 years amongst the males (range: 50-73). Eight of these participants were African while only 2 were Caucasian. The histological subtypes, steroid hormone receptor status (estrogen receptor and progesterone receptor) and HER-2/neu status was determined by the National Health Laboratory Services with the histologic types being defined according to the WHO classification.

Patients suffering from breast cancer were categorised into a surgical (modified radical mastectomy and lumpectomy), radiotherapy and chemotherapy/tamoxifen group, while the oesophageal cancer patients were categorised into surgical, radiotherapy or chemotherapy groups.

The majority of breast cancer patients received surgical intervention (19 patients) while the rest (6 patients) received neo-adjuvant chemotherapeutic treatment in combination with hormonal treatment (Tamoxifen). None of the breast cancer participants received radiotherapy as a first-line treatment.

Eight out of the 10 oesophageal cancer patients received palliative radiotherapy in conjunction with recurrent oesophageal dilatation or the insertion of an oesophageal stent (e.g. Procto-livingston tube), and the remaining 2 received major surgical intervention including a 3 stage oesophagectomy with gastric pull-up and a transthoracic-partial-oesophago-gastrectomy.

None of the patients had any history of autoimmune disease or recent infection. Information regarding the clinicopathological characteristics of all patients is summarised in Table 3.1.

Table 3.1: Clinicopathological characteristics of the patients		
	Breast cancer	**Oesophageal cancer**
Total patients recruited (N)	25	10
Gender		
1. Females	25	7
2. Males	0	3
Mean age		
1. Females	59	62
2. Males	0	61
Race		
1. African	17	8
2. Caucasian	8	2
Histological diagnosis		
1. Infiltrating ductal carcinoma	21	N/A
2. Infiltrating lobular carcinoma	3	N/A
3. Mucinous	1	N/A
3. Squamous cell carcinoma	N/A	9
4. Adenocarcinoma carcinoma	N/A	1
Stage of disease		
Stage 1	4	0

	Breast cancer	**Oesophageal cancer**
Stage 2	5	0
Stage 3	10	6
Stage 4	6	4
Treatment		
1. Surgery only	19	2
2. Chemotherapy and Tamoxifen	6	0
3. Radiotherapy only	0	8
Number of participants who died before 7 weeks post-treatment	2	6
Number of patients who were still alive at 7 weeks post-treatment	19	0
Number of patients who were lost to follow-up at 7 weeks post-treatment	4	4

4 Materials and methods

4.1 Flow cytometric analysis

Whole blood (5ml) was drawn from each participant in an EDTA tube at specified time points including:

vi. Before treatment,

vii. Day 1 after treatment,

viii. 5-7 weeks post-treatment.

After sample collection the whole blood was stained using 2 staining techniques with fluorescent-tagged monoclonal antibodies including:

- *Intracellular*: FoxP3, CD25, CD4, CD8, CD127, CD45 and,
- *Extracellular:* CD25, CD4, CD8, CD127, CD45.

This is in light of the fact that the identification of Treg cell populations with intracellular staining techniques is a time consuming and technically challenging process, and sufficient identification of Treg cell populations ($CD4^{+}CD25^{+}CD127^{low}$), in the absence of $FoxP3^{+}$, has previously been demonstrated (Decker et al. 2012). T-lymphocyte subsets were identified using a Gallios flow cytometer (Beckman Coulter, Miami, FL), and each sample was run in duplicate to ensure the consistency of the results and then averaged to give a final result. Due to the limited frequency of Treg cells in the peripheral blood, a minimum of 100 000 events were recorded for each sample. Kaluza software (Beckman Coulter 2010, Miami, FL) was used for the digital compensation and analysis of results.

4.2 Monoclonal antibodies

4.2.1 Intracellular staining

The following monoclonal antibodies were used: CD4-FITC (Beckman Coulter), CD127-PC5 (Beckman Coulter), CD45-Pacific Blue (Beckman Coulter), Human Regulatory T-cell staining kit containing FoxP3-PE, CD4-FITC, CD25-APC (eBioscience, Hatfield, UK). Staining of samples was performed as specified by the manufacturer. Briefly, 100µl of whole blood was stained with surface antibodies (CD45 (10µl), CD4 (10µl), CD25 (10µl), CD127 (10µl), CD8 (5µl)) and incubated in the dark, on ice for 30 minutes. FoxP3 Fixation/Permeabilization buffer (1:4 conc) (1ml) was added to the sample, which was then incubated in the dark on ice for an additional 30 minutes. FoxP3 normal rat serum (2µl) was added to sample and incubated for 15 minutes at room temperature. FoxP3 (10µl) monoclonal antibody was added to the sample, which was again followed by a 30 minute incubation period in the dark. Flow count fluorosphere beads (Beckman Coulter) (100µl) were added to the sample immediately before acquisition.

4.2.2 Extracellular staining

The following monoclonal antibodies were used: CD8-PC7 (Beckman Coulter), CD4-FITC (Beckman Coulter), CD127-PC5 (Beckman Coulter), CD45-Pacific Blue (Beckman Coulter), and CD25-APC (Biolegend, San Diego, CA). Staining of samples was performed as specified by the manufacturer. Briefly, 100µl of whole blood, in the presence of monoclonal antibodies (CD45 (10µl), CD4 (10µl), CD25 (10µl), CD127 (10µl), CD8 (5µl)), was incubated in the dark, on ice for 30 minutes. The red blood cells were lysed and fixed using the automated TQ Prep (Beckman Coulter) sample processor. Flow count fluorosphere beads (Beckman Coulter) (100µl) were added to the sample and events were immediately acquired using the

flow cytometer. Prior to each patient acquisition, flow-check fluorescence (Beckman Coulter) was run to verify the optical alignment.

The spill-over coefficients of the 6 fluorochromes was determined by using strongly positive single-colour monoclonal antibodies in separate tubes for each of the fluorochromes: FITC, PE, APC, PC5, APC7 and PB (Carrock et al. 1997). Thereafter, these compensation standards for the lymphocyte population were used to derive a compensation matrix with Kaluza software (Beckman Coulter). $CD4^+$ and $CD8^+$ T-lymphocytes were quantified as a percentage of the total lymphocyte population while Treg T-lymphocytes populations were quantified as a percentage of total $CD4^+$ $CD45^+$ and $CD8^+CD45^+$ T-lymphocyte subsets at each time point. Flow count fluorosphere beads (Beckman Coulter) were used to determine the concentration (cells/µl) for the counting of specific T-lymphocyte populations.

4.3 Gating strategy

4.3.1 Step 1: Identification of lymphocyte population

The lymphocyte population was identified using the CD45 fluorescence (CD45) versus the side light-scatter (SS). This approach has been suggested as a more accurate method for lymphocyte identification as compared to the more conventional light scatter gating (forward scatter versus side-scatter-approach) as it reduces the likelihood of non-lymphocyte contamination within the gate (Nicholson et al. 1996, Storie 2005). Lymphocytes were characterised as $CD45^+$ bright, with low side scatter, (Figure 4.3 A).

4.3.2 Step 2: Identification of total $CD4^+$ ($CD4^+CD45^+$) and total $CD8^+$ ($CD8^+CD45^+$) T-lymphocyte populations

Sequential gating of the total $CD45^+$ T-lymphocyte population (Figure 4.3 A: N) was used to identify the $CD4^+$ and $CD8^+$ T-lymphocytes subsets (Figure 4.3 B). These cell population represent the total $CD4^+$ T-cell ($CD4^+CD45^+$) population and total $CD8^+$ T-cells ($CD8^+CD45^+$).

4.3.3 Step 3: Identification of $CD25^+$ expression by $CD4^+$ T-lymphocyte and $CD8^+$ T-lymphocyte

The frequency of CD25-expressing T-lymphocyte population was identified by sequentially gating on $CD45^+$ lymphocyte region (N). $CD4^+$ (Figure 4.3 C) and $CD8^+$ T-cells were gated on region N (Figure 4.3 D). The $CD4^+$ and $CD25^+$ cells were gated on the $CD45^+$ T-lymphocytes. This cell subset will be referred to as $CD4^+CD25^{+/-}$ and $CD8^+CD25^{+/-}$ in the remainder of the text.

4.3.4 Step 4: Identification of $CD4^+$ and $CD8^+$ regulatory T-lymphocytes

Treg cells were identified by three different gating strategies:

1. Firstly, Treg cells were characterised as $CD4^+$ (Figure 4.3 E) or $CD8^+$ T-lymphocytes (Figure 4.3 F) which co-expressed $CD127^+$ and $CD25^+$ receptors (Simonetta et al. 2010).
2. Secondly, Treg cells were characterized as $CD4^+$ (Figure 4.3 G) or $CD8^+$ T-lymphocytes (Figure 4.3 H), which co-expressed $CD25^+$, surface marker and intracellular $FoxP3^+$.
3. Lastly, Treg cells were characterized as $CD4^+$ (Figure 4.3 I) or $CD8^+$ T-lymphocyte (Figure 4.3 J) which co-expressed $CD127^+$ surface marker and intracellular $FoxP3^+$.

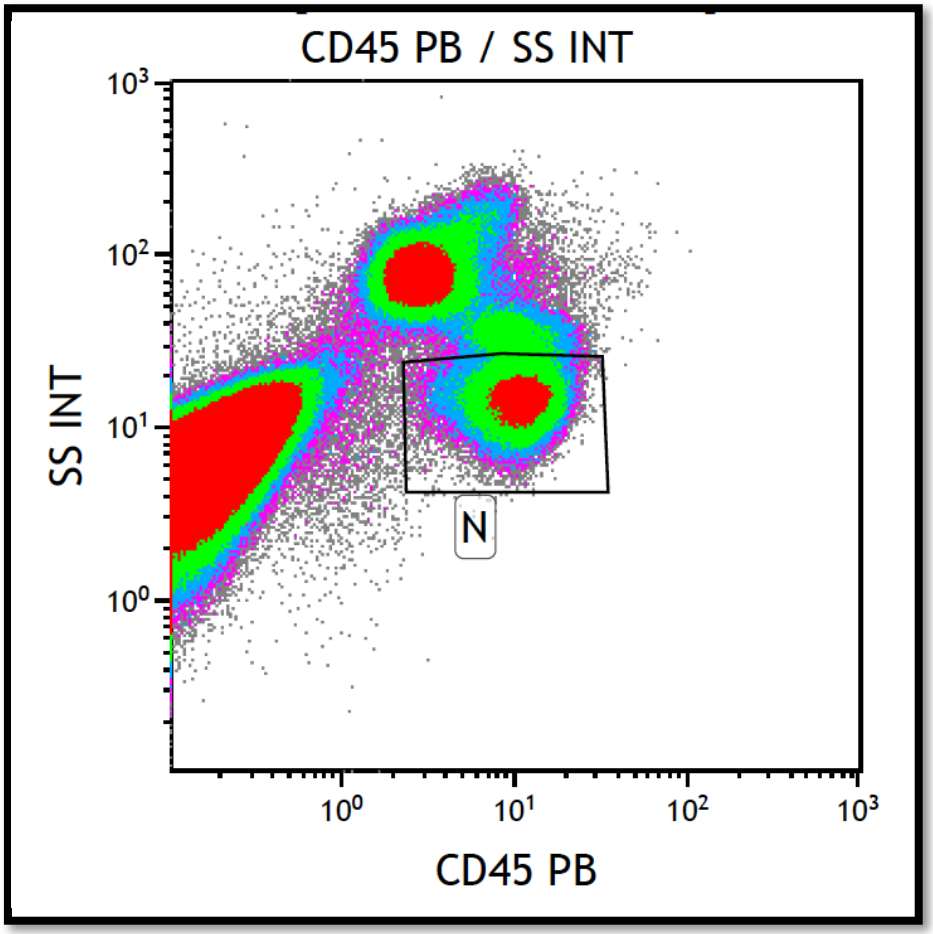


Figure 4.3 A: Total lymphocyte population. Figure representative of *n* samples.

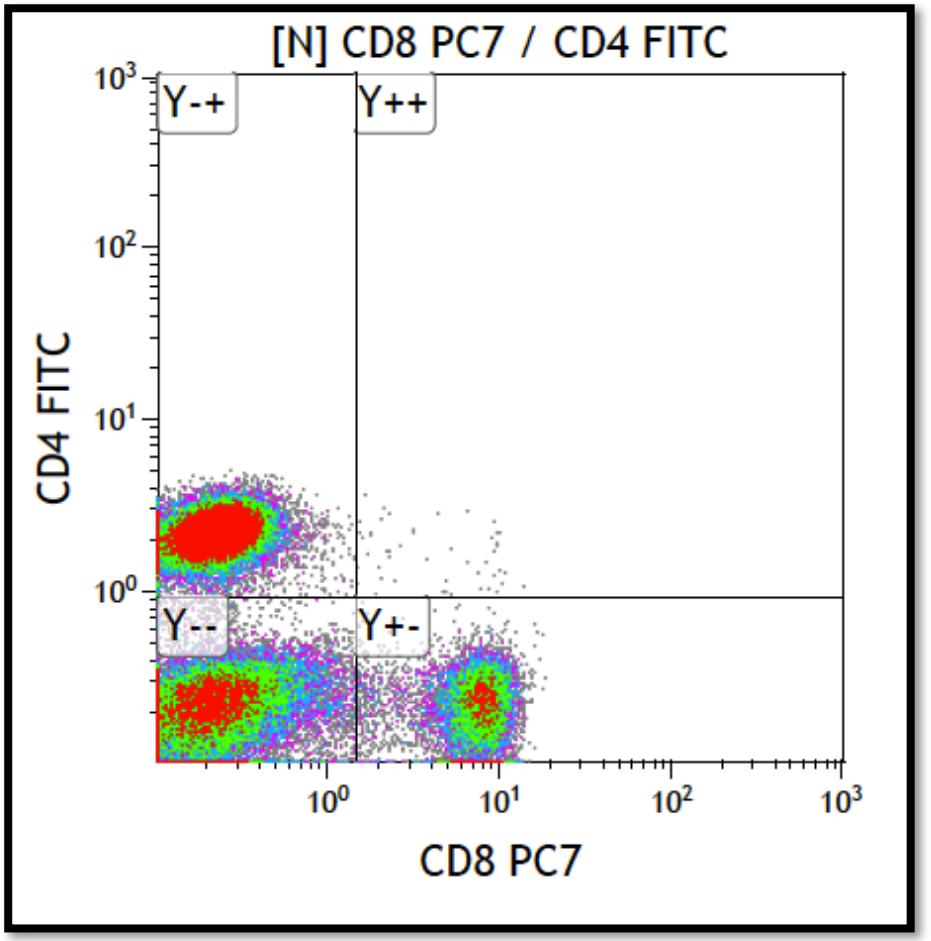


Figure 4.3 B: Total $CD4^+$ T-lymphocyte and $CD8^+$ T-lymphocyte. Figure representative of *n* samples.

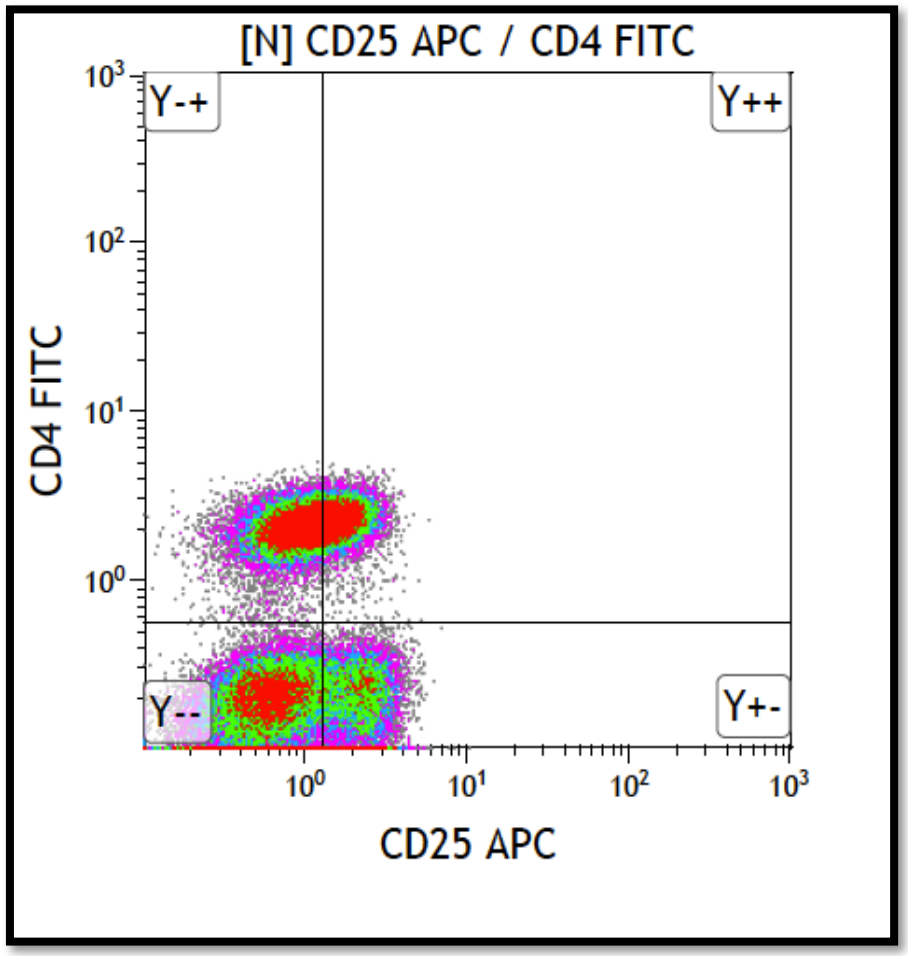


Figure 4.3 C: CD25$^+$ expression on CD4$^+$ T-lymphocyte. Figure representative of *n* samples.

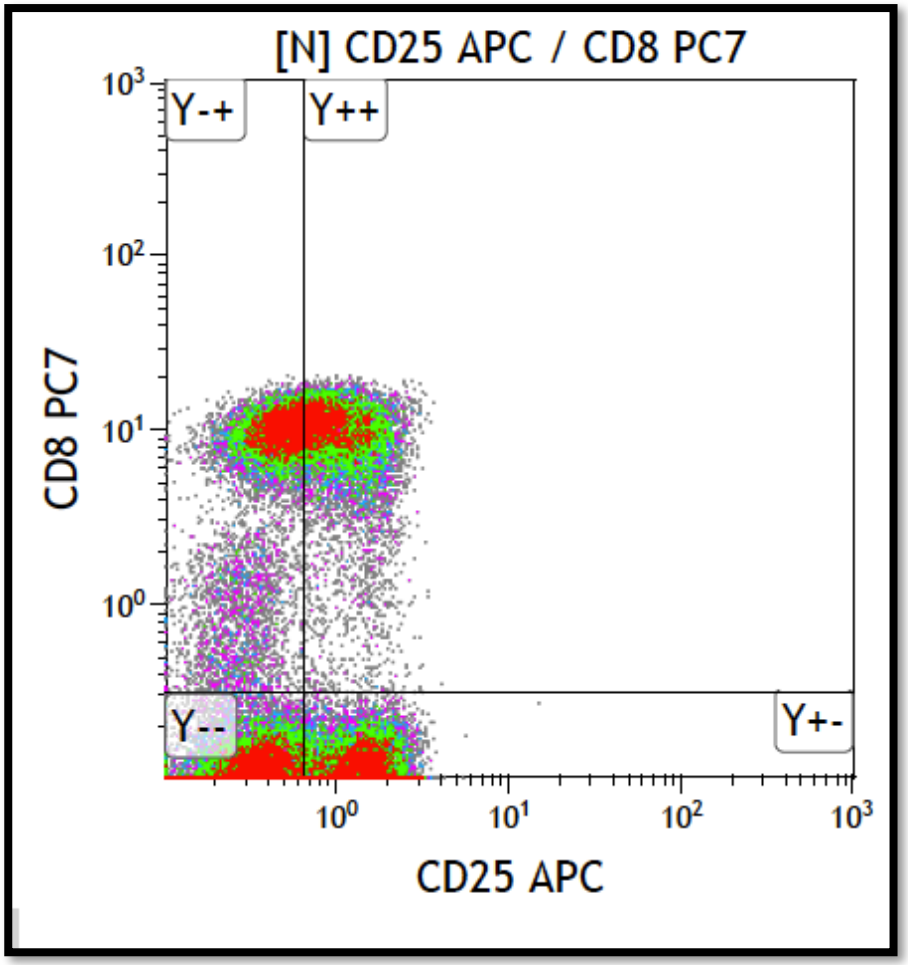


Figure 4.3 D: CD25$^+$ expression on CD8$^+$ T-lymphocyte. Figure representative of *n* samples.

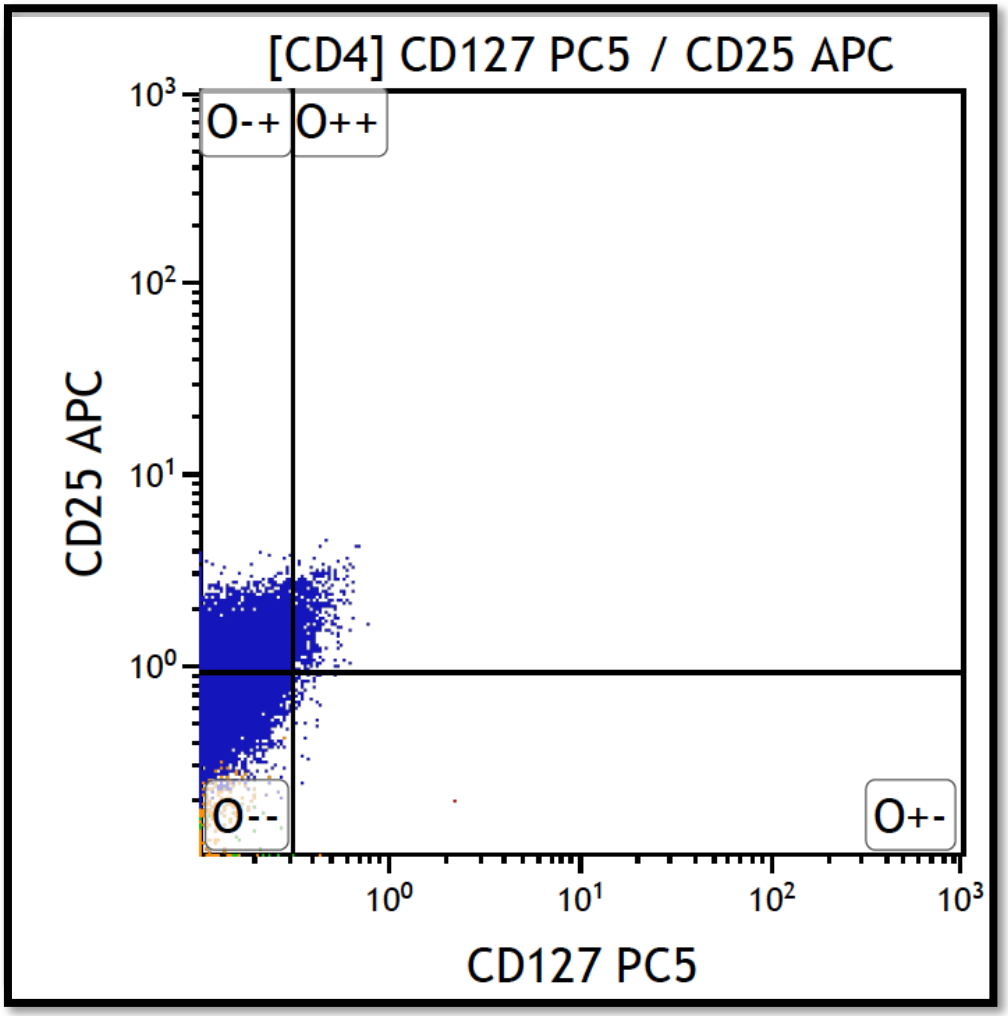


Figure 4.3 E: $CD4^+CD25^+CD127^+$ Treg cell population. Figure representative of *n* samples.

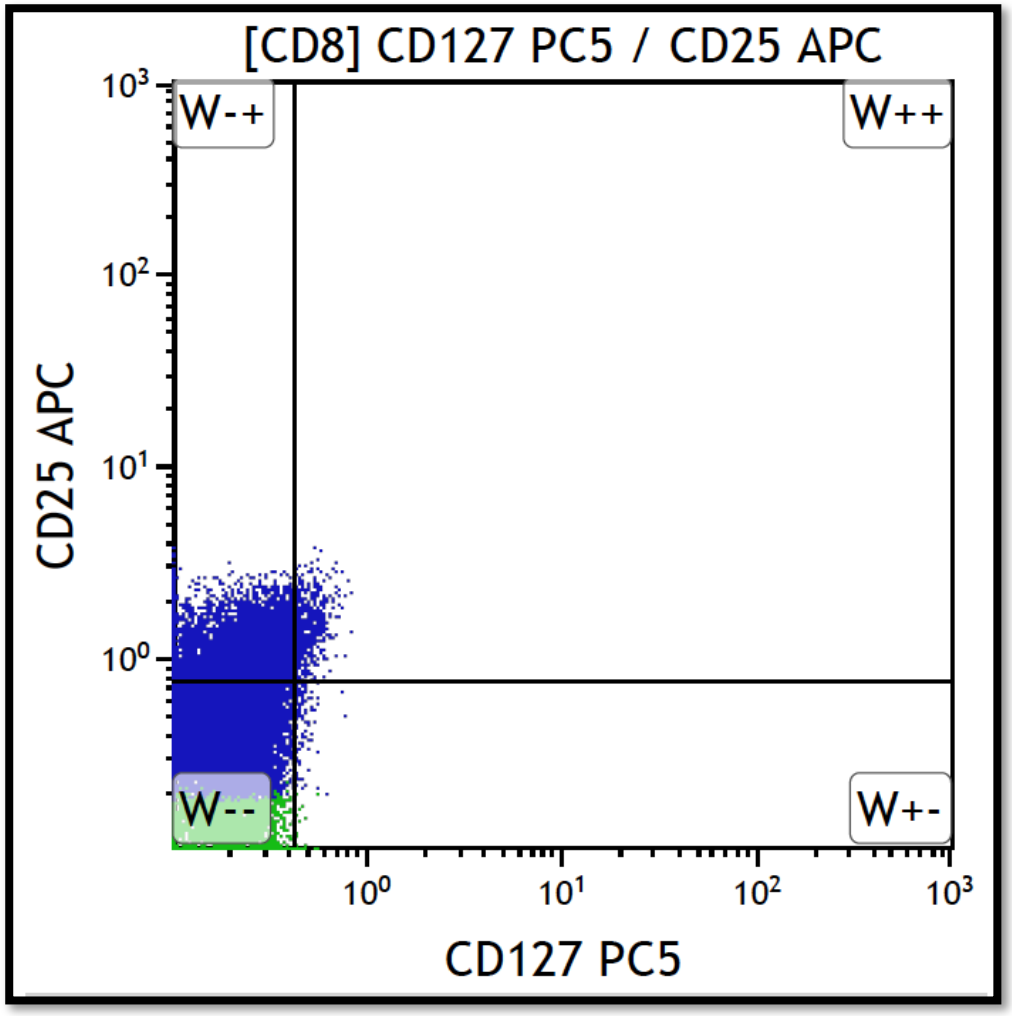


Figure 4.3 F: $CD8^+CD25^+CD127^+$ Treg cell population. Figure representative of *n* samples.

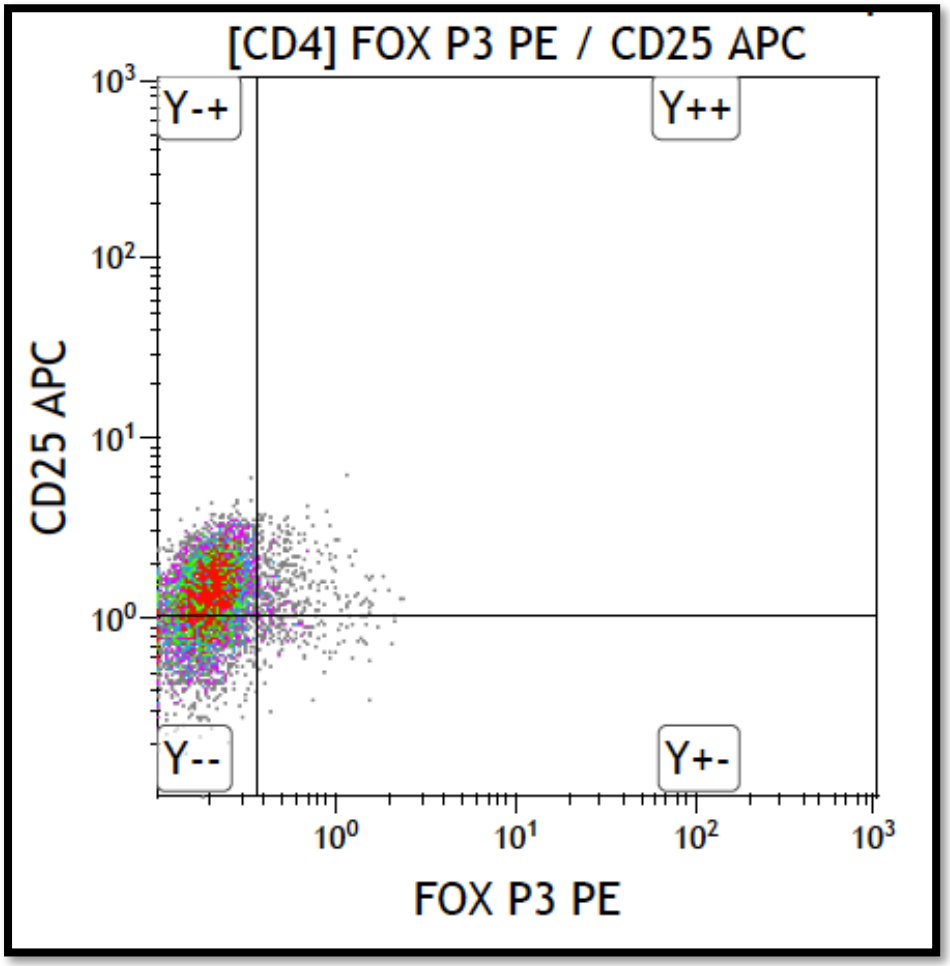


Figure 4.3 G: $CD4^+CD25^+FoxP3^+$ Treg cell population. Figure representative of *n* samples.

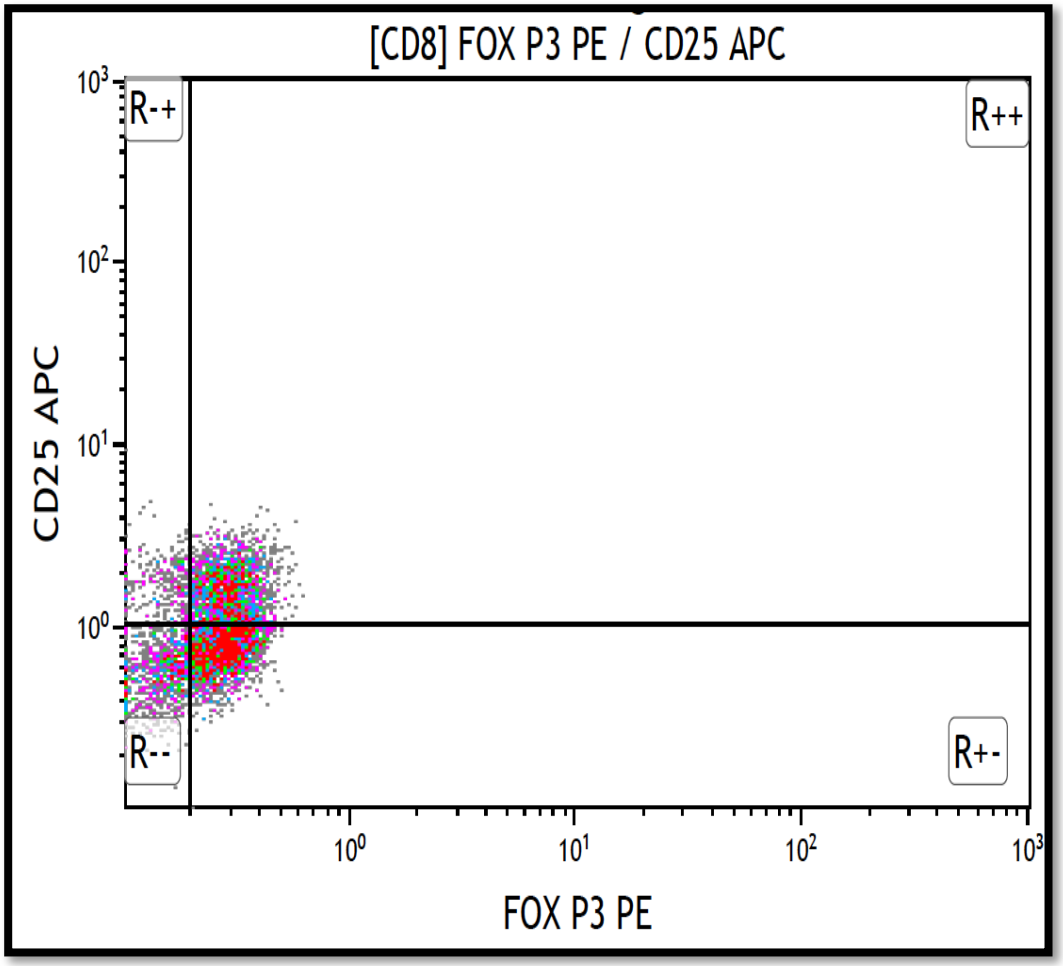


Figure 4.3 H: $CD8^+CD25^+FoxP3^+$ Treg cell population. Figure representative of *n* samples.

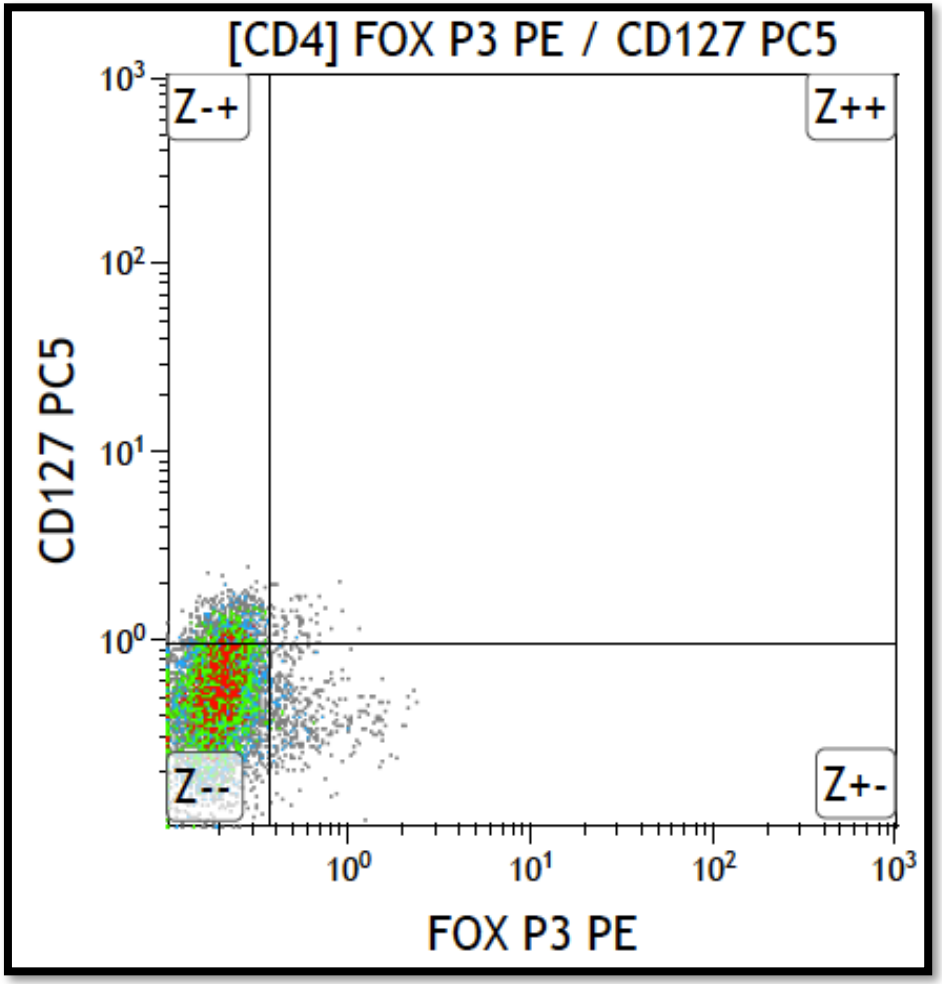


Figure 4.3 I: $CD4^+CD127^+FoxP3^+$ Treg cell population Figure representative of *n* samples.

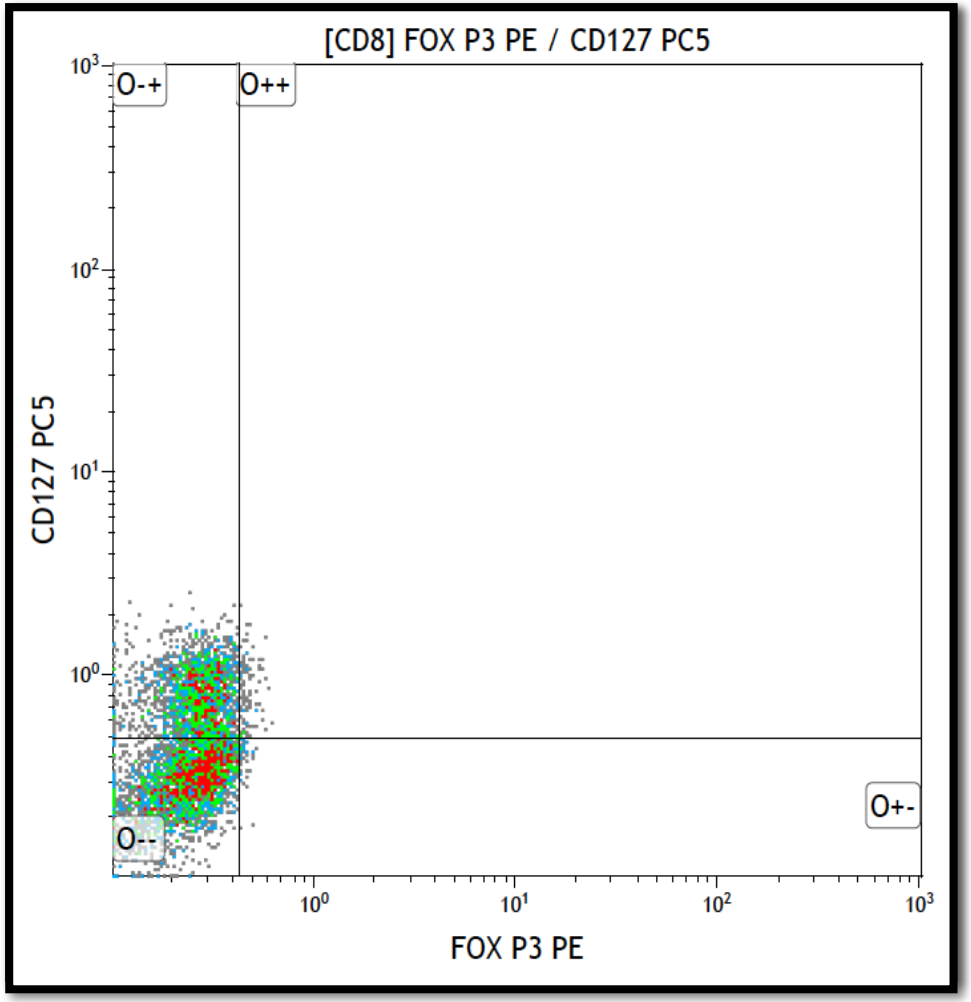


Figure 4.3 J: $CD8^+CD127^+FoxP3^+$ Treg cell population . Figure representative of *n* samples.

4.4 Statistical analysis

Statistical analysis was undertaken using STATA version 13 (Statacorp. 2 Station, TX: StataCorp LP). All statistical comparisons between the clinica patients were analysed using an unpaired Student t-test, Chi-squared test and I test, as appropriate, and results are reported as mean ± standard deviation (confidence interval. The association between immunological levels and categor were analysed with the non-parametric Wilcoxon rank-sum test and ANO association between repeated immunological measurements from the sample carried out using the Wilcoxon sign-rank (matched pairs) statistic, and results a median ± interquartile range (IQR). A P-value of <0.05 was considered a significant.

5 T-lymphocyte subsets ($CD4^+$, $CD8^+$ and Treg cells) in breast and oesophageal cancer and association with stage of disease

5.1 Introduction

Within the South African healthcare setting, affliction with breast or oesophageal cancer is associated with significant morbidity and mortality. Numerous factors have been shown to contribute to the poor outcome of these patients, with advanced stage of disease at presentation playing a substantial role in a large proportion of these cancer-associated mortalities (American Cancer Society 2011, Kingham et al. 2013). Late presentation to healthcare facilities limits the treatment options available and consequently diminishes patient survival. This chapter focuses on the clinicopathological distribution and immune profile of 35 patients presenting with breast and oesophageal cancer at Steve Biko Academic Hospital and Kalafong Hospital between March 2012-August 2012.

5.2 Results

5.2.1 Determination of stage of disease at first presentation in patients suffering from breast or oesophageal cancer

The majority of participants diagnosed with cancer who were eligible for the study presented with stage 3 or stage 4 disease, with less than 20% of cancer patients presenting with stage 1 or stage 2 disease (Figure 5.1). This analysis was statistically significant (P=0.028). All patients suffering from oesophageal cancer presented at a late disease stage (stage 3 and 4) (as described in Chapter 3: Table 3.1). The analysis of patients presenting with breast cancer showed that a statistically significant number of African patients (88%) presented with stage 3 and 4 disease compared to Caucasian patients (12%), P=0.001 (Figure 5.2).

Squamous cell carcinoma was the most common histopathological subtype (90%) of oesophageal cancer noted in this setting (as described in Chapter 3: Table 3.1), which correlates with other previously described African (Tettey et al. 2013) and Asian studies (Zhu et al. 2011). Eighty four percent of participants presenting with breast cancer presented with a histopathological diagnosis of infiltrating ductal carcinoma (as described in Chapter 3: Table 3.1), with infiltrating lobular carcinoma and mucinous carcinoma contributing the remaining 16%. Despite the late presentation of the majority of patients presenting with breast cancer, mortality was significantly less (P=0.0004) than that seen in the oesophageal cancer cohort (60%), with 8% of breast cancer participants demising within 3 months of diagnosis.

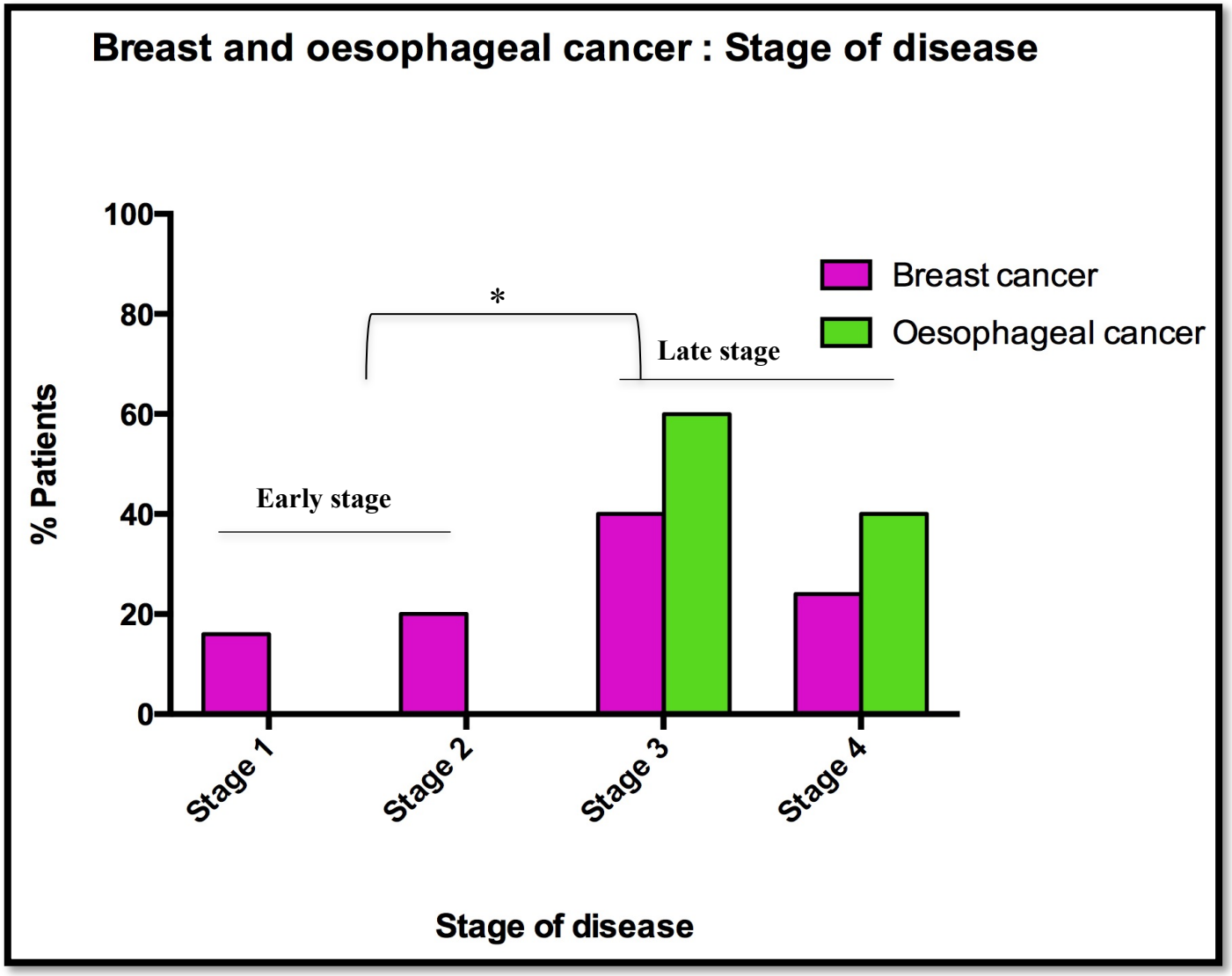


Figure 5.1: The distribution of breast and oesophageal cancer patients and stage of disease at presentation. This Figure represents the frequency distribution of breast and oesophageal cancer patients in the different stages of disease, at presentation. Less than 20% of patients presented with stage 1 disease, with only breast cancer patients presenting in the early stages of disease (stage 1 or stage 2) (P=0.028.) All oesophageal cancer patients presented with stage 3 or stage 4 disease.
(NS =not significant, *P<0.05, **P<0.01, ***P<0.001)

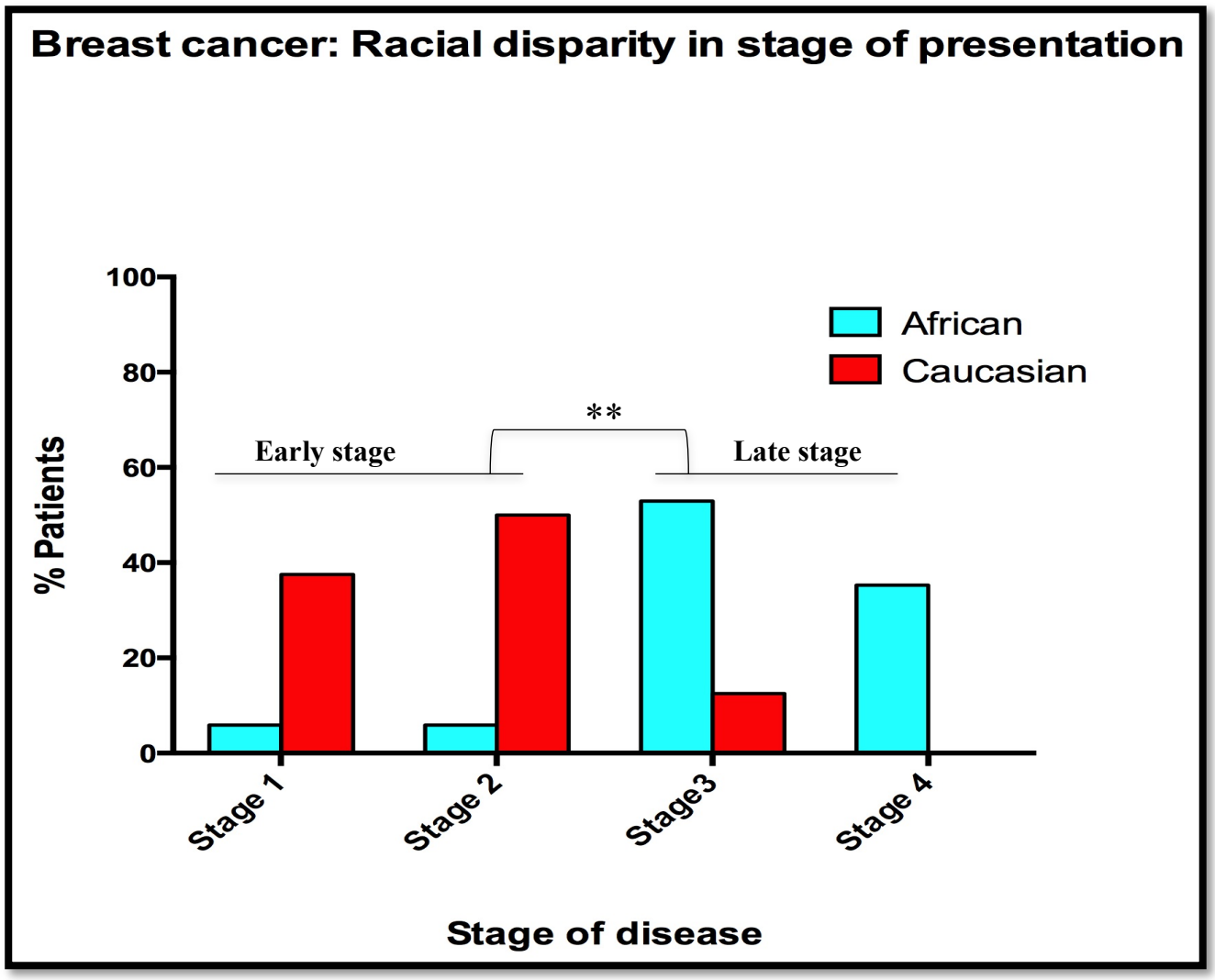


Figure 5.2: The distribution of breast cancer according to race and stage of disease at presentation. This Figure represents the frequency distribution of African and Caucasian patients at different stages of disease, at presentation. 88 % of African patients presented with late stage (stage 3 or 4) disease, compared to 12% of Caucasian patients (P=0.001). (NS =not significant, *p<0.05, **p<0.01, ***p<0.001).

5.2.2 The prevalence of $CD4^+CD25^+$ and $CD8^+CD25^+$ T-lymphocyte cell populations in breast and oesophageal cancer patients

The included participants were evaluated for signs of immune suppression through the quantification of the circulating T-lymphocyte subsets. The circulating total CD4% in breast and oesophageal cancer patients was compared to a previously described reference ranges drawn from clinically healthy volunteers in the Gauteng region (Lawrie et al. 2009). This range was found to be between 27.7- 58%. Normal levels of CD8% has been described as 13-42% with a $CD4^+/C8^+$ T-cell ratio of 1.4-1.66 being considered as normal (Bofill et al. 1992, CDC 2004).

Cancer patients were found to have a median total $CD4^+$ T-lymphocyte percentage, defined as $CD4^+CD45^+$, of 48.4% (95% Confidence Interval (CI) 43.3%-52.2%) which falls within the the normal range despite the late stage of cancer presentation (Figure. 5.3). However, the total $CD4^+$ concentration ($CD4^+$ count) was found to be lower in this cohort (246 cells/µl (95% CI 210-399 cells/µl)) , as compared to levels described previously in healthy volunteers (500-2010 cells/µl) in the Gauteng region (Lawrie et al. 2009).

The median total $CD8^+$ T-lymphocyte percentage, defined as $CD8^+CD45^+$, was determined as 22.43% (95% CI 20.03-26.36), which fell within normal limits. However, the total $CD8^+$ T-cell concentration ($CD8^+$ count) was also found to be lower (141 cells/µl, (95% CI 111-190)) in this cohort as compared to that observed in healthy volunteers (209-924 cells/µl). The $CD4^+/CD8^+$ T-cell ratio was calculated to be 2 (95% CI 1.46-2.5), which is >1 and is thus considered as normal. Interestingly, a large proportion of $CD4^+$ (89%; P=0.0017) and $CD8^+$ (57.2%; P=0.009) T-lymphocytes also expressed $CD25^+$ surface marker (Figure. 5.3).

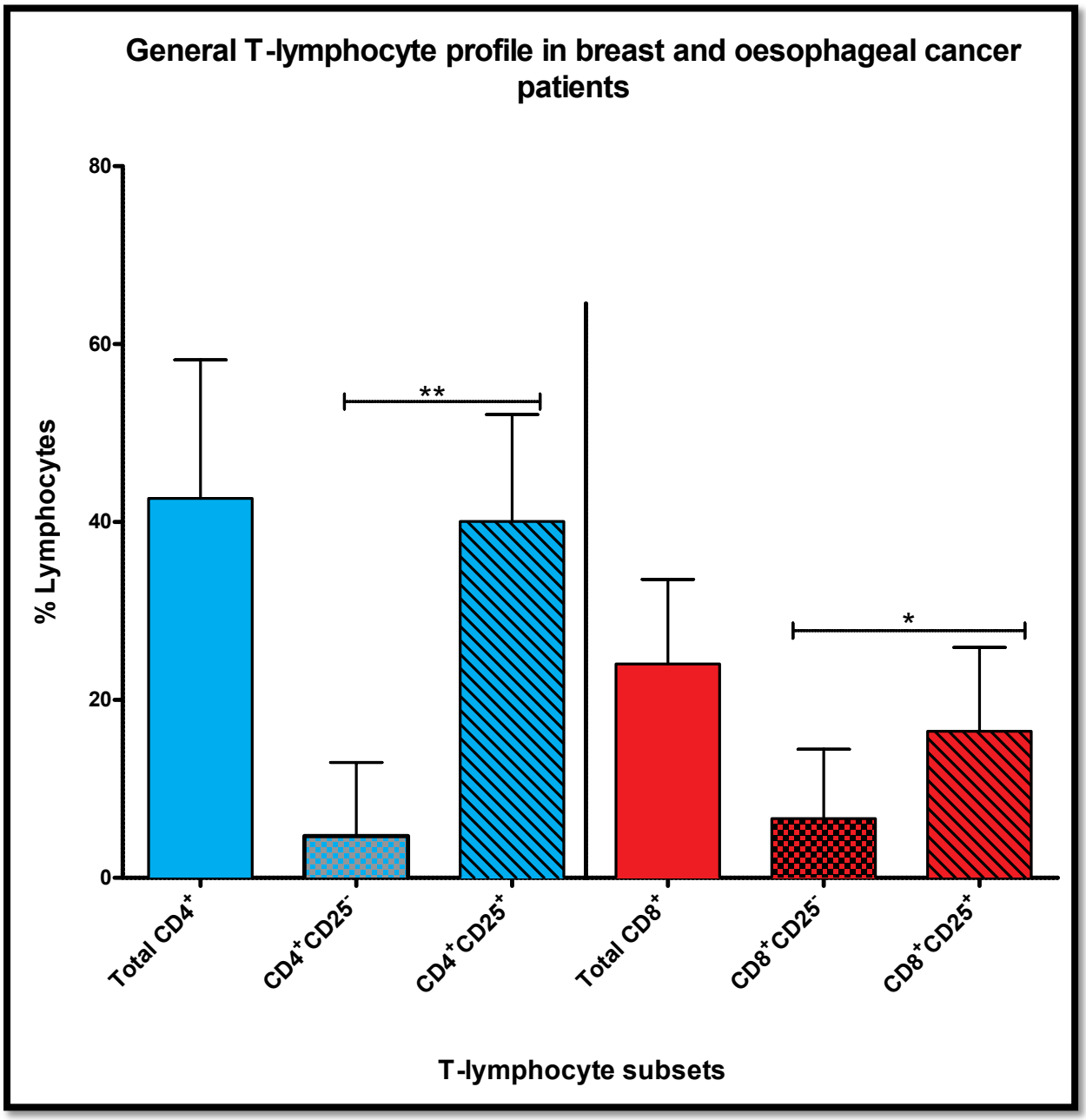


Figure 5.3: The $CD4^+$ and $CD8^+$ T-lymphocyte subsets of breast and oesophageal cancer patients at first presentation. The total $CD4^+$, $CD4^+CD25^-$, $CD4^+CD25^+$, total $CD8^+$, $CD8^+CD25^-$, $CD8^+CD25^+$ T-lymphocytes percentage of patients presenting with breast and oesophageal cancer are shown as the median % ± IQR. The median total $CD4^+$ and total $CD8^+$ lymphocyte concentration and IQR at presentation are 246 cells/µl (IQR: 133-566) and 141 cells/µl (IQR:56-306). (NS =not significant, *P<0.05, **P<0.01, ***P<0.001)

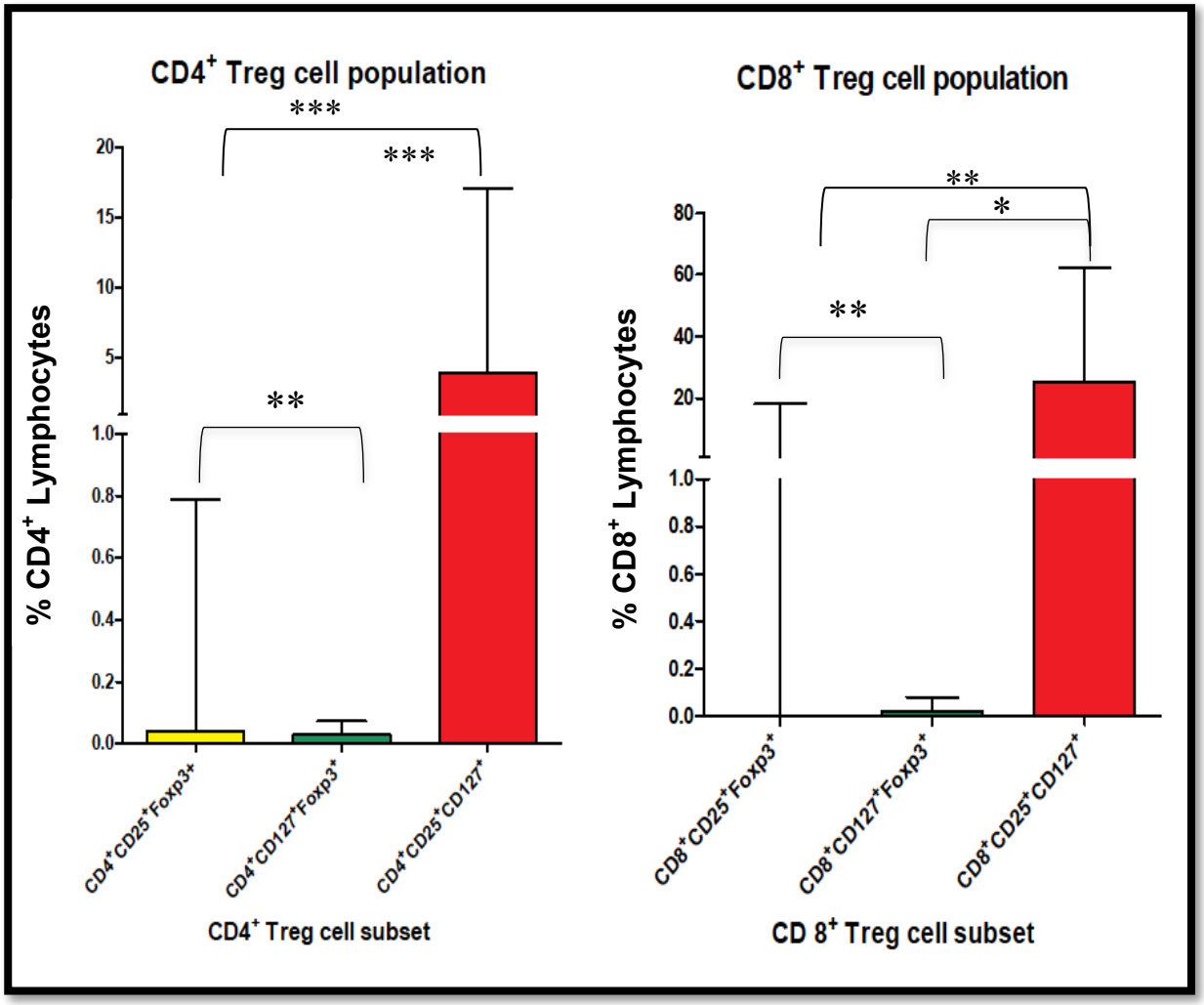


Figure 5.4: The distribution of circulating $CD4^+$ and $CD8^+$ T regulatory cell subsets of breast and oesophageal cancer patients at first presentation. The $CD4^+$ and $CD8^+$ Treg cell subsets are presented as the median % ± IQR. (NS =not significant, *P<0.05, **P<0.001, ***P<0.0001)

5.2.4 Association between circulating levels of $CD4^+$ and $CD8^+$ T-lymphocytes and pathological stage of breast cancer and oesophageal cancer

To explore the relationship between the frequency of peripherally circulating $CD4^+$ and $CD8^+$ T-lymphocytes and pathological stage of disease in breast and oesophageal cancer, whole blood was drawn from participants before receipt of antitumor treatment and was analysed for the frequency of $CD4^+CD25^-$, $CD4^+CD25^+$, $CD8^+CD25^-$ and $CD8^+CD25^+$ T-lymphocytes subsets. Although previous studies have assessed Treg cell status as those co-expressing $CD4^+$ and $CD25^+$ surface markers, a large proportion of these cells have been shown to not display suppressive activity and are regarded as activated T-lymphocytes (Stanzani et al. 2004).

A statistically significant variation in the circulating levels of $CD4^+CD25^+$ T-lymphocytes was observed in cancer patients at different stages of disease at presentation (Figure 5.5) (P=0.0572); however, pairwise comparison did not show a significant difference between the different stages of disease. The circulating levels of $CD8^+CD25^+$ T-lymphocytes were also increased as compared to $CD8^+CD25^-$ T-lymphocytes, however it did not reach statistical significance (Figure 5.5).

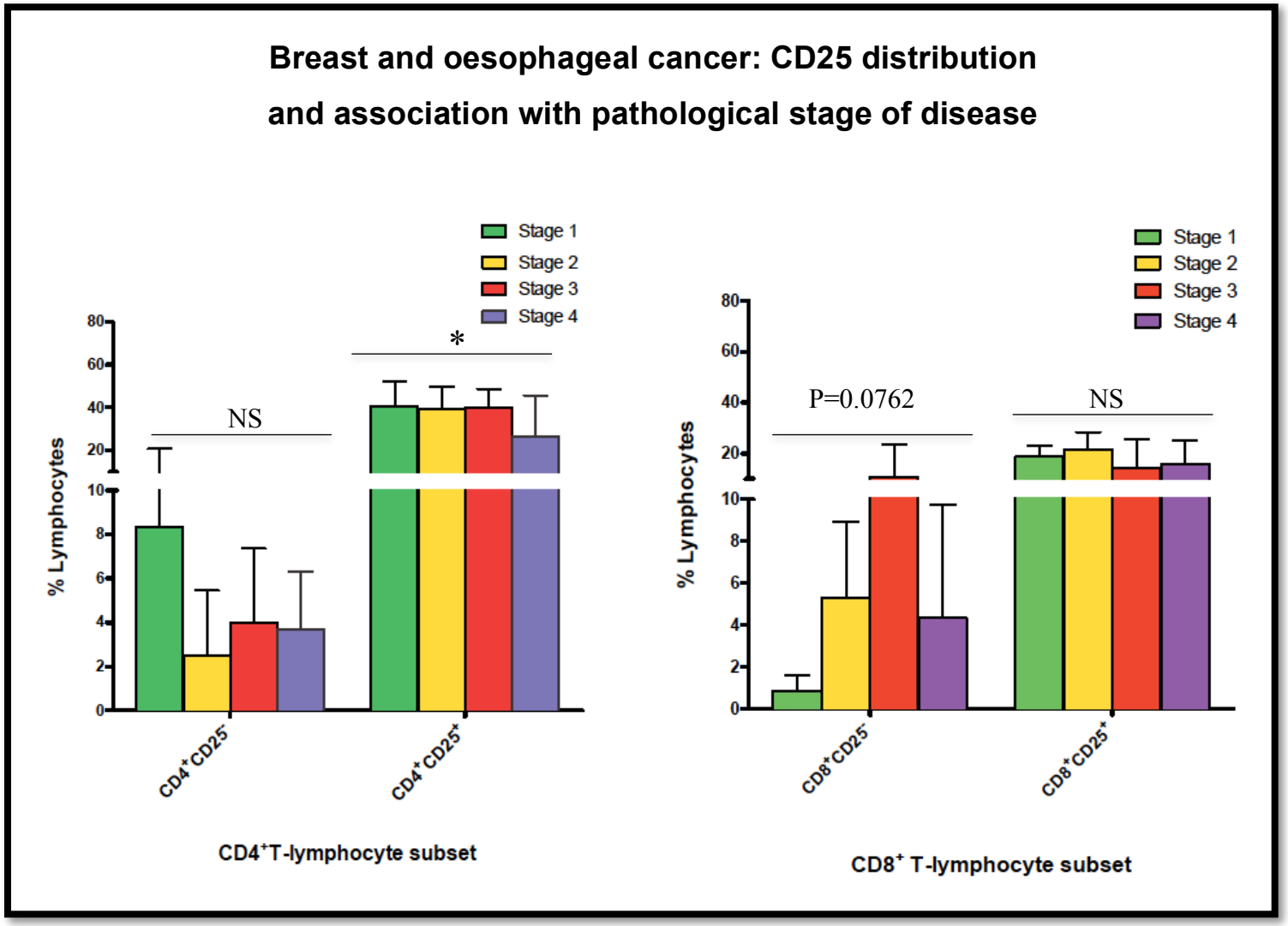


Figure 5.5: The distribution of CD25 in $CD4^+$ and $CD8^+$ T-lymphocyte populations in breast and oesophageal cancer patients according to stage of disease progression at presentation. The $CD4^+$ and $CD8^+$ T-lymphocyte subsets expressing $CD25^{+/-}$ are presented as the median ± IQR. (NS =not significant, *p<0.05, **p<0.01, ***p<0.001)

5.2.5 Association between circulating levels of $CD4^+$ and $CD8^+$ T-lymphocytes and pathological stage of breast cancer

Patients presenting with breast cancer were staged according to the American Joint Committee on Cancer (AJCC) TNM staging and then further subdivided into 4 stages depending on their TNM status (stage I-IV). Breast cancer patients were found to have a substantially higher proportion of circulating $CD25^+$ expressing $CD4^+$, and $CD8^+$ T-lymphocytes (Figure 5.6), as compared to $CD4^+CD25^-$ and $CD8^+CD25^-$ T-lymphocytes. A significantly higher circulating level of $CD25^+$ expressing $CD4^+$ T-cells as compared to $CD4^+CD25^-$ T-lymphocytes observed in patients presenting with stage 3 disease (P=0.0039) (Figure 5.6). A significant variation was observed in the frequency of $CD4^+CD25^-$ T-lymphocytes (P=0.021) in breast cancer patients presenting at different stages of disease, however pairwise comparison did not yield a significant result (Figure 5.6). This may be due to the small sample size in each "disease stage" group.

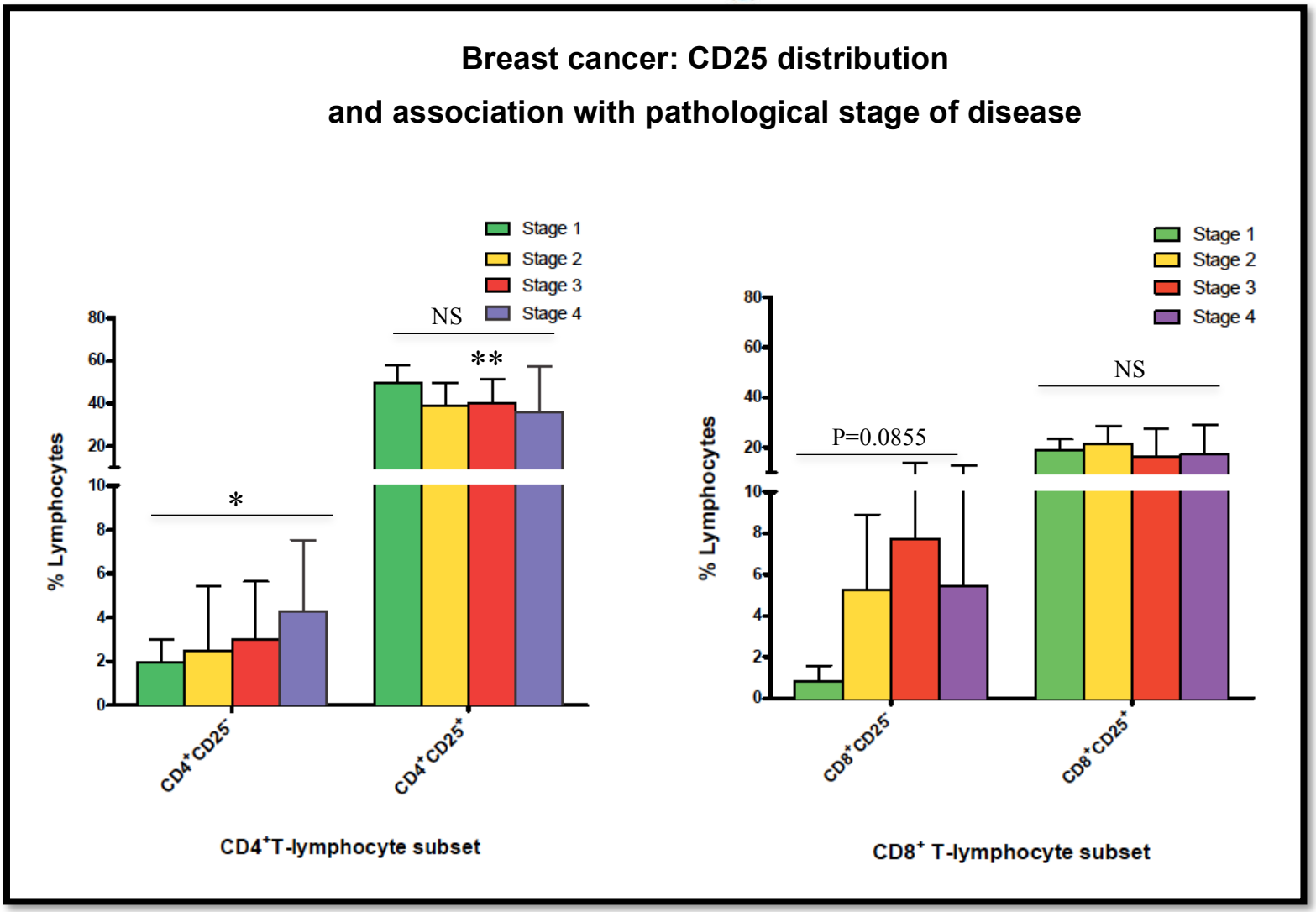


Figure: 5.6. The distribution of CD25 in $CD4^+$ and $CD8^+$ T-lymphocyte populations in patients presenting with breast cancer, according to stage of disease progression at first presentation. The $CD4^+$ and $CD8^+$ T-lymphocyte subsets expressing $CD25^{+/-}$ are presented as the median % ± IQR. (NS =not significant, *P<0.05, **P<0.01, ***P<0.001)

5.2.6 Association between circulating levels of $CD4^+$ and $CD8^+$ T-lymphocytes and pathological stage of oesophageal cancer

Patients presenting with oesophageal carcinoma were also staged according to the American Joint Committee on Cancer (AJCC) TNMG staging and then further subdivided into 4 groups depending on their TNMG status (American Cancer Society 2013). Elevated levels of $CD4^+CD25^-$ and $CD8^+CD25^-$ T-lymphocytes were observed in patients presenting with stage 3 disease as compared to stage 4 disease (Figure 5.7); however, this did not reach significance (P=0.3938 and P=0.6698). Patients presenting with stage 4 disease displayed significantly higher circulating percentage of $CD4^+CD25^+$ T-lymphocytes (P=0.0550) and $CD8^+CD25^+$ (P=0.0550), as compared to patients presenting with stage 3 disease (Figure 5.7).

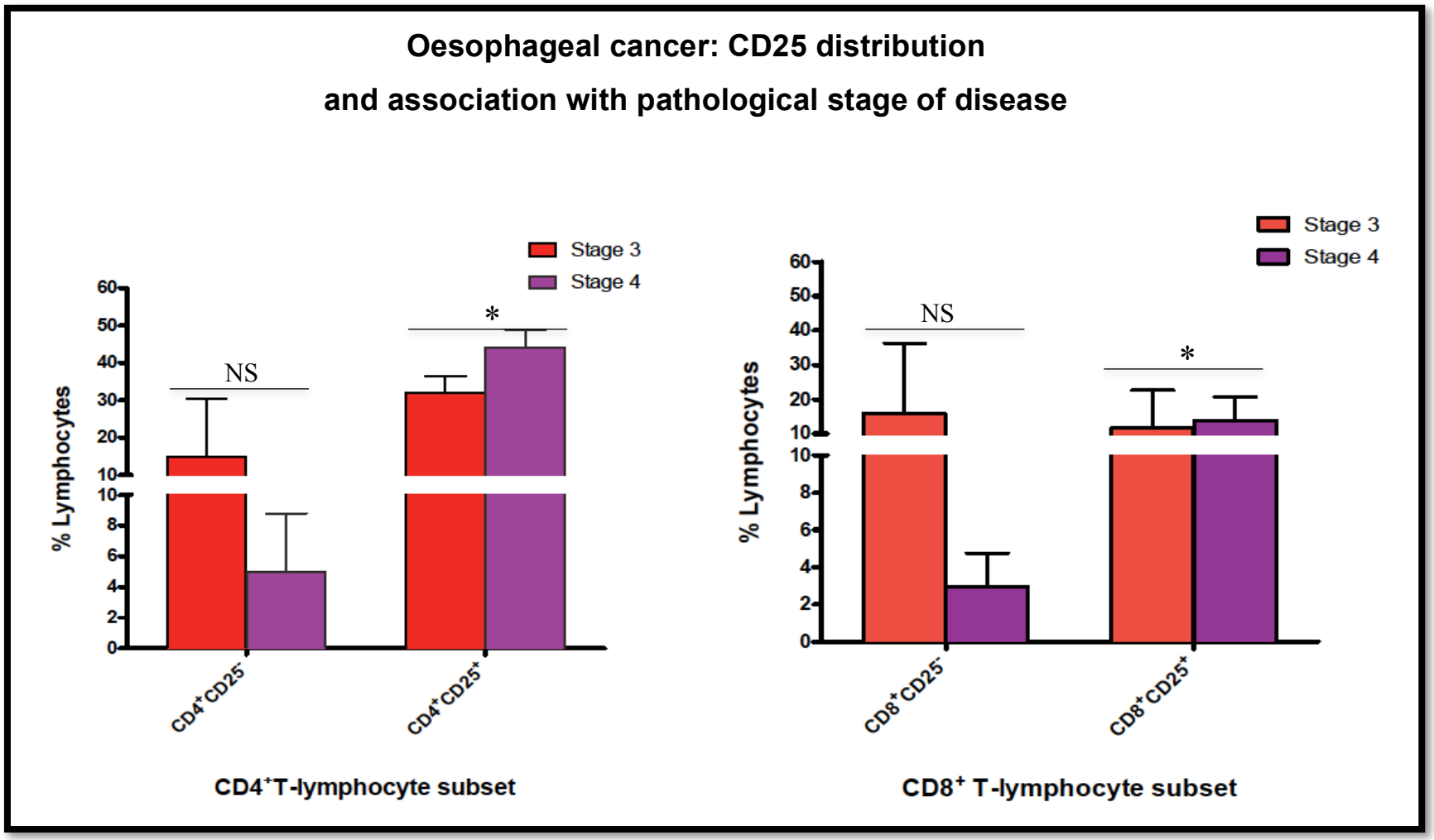


Figure: 5.7 The distribution of CD25 in $CD4^+$ and $CD8^+$ T-cell subsets in patients presenting with oesophageal cancer, according to stage of disease progression at first presentation. The expression of $CD25^{+/-}$ by $CD4^+$ and $CD8^+$ T-lymphocytes are presented as the median% ± IQR.

5.2.7 Intracellular versus extracellular staining

In order to explore the benefits of characterising and measuring circulating Treg cell subsets, with an extracellular staining technique, as opposed to the conventional combination of both intracellular and extracellular staining technique, blood samples from breast and oesophageal cancer patients were stained using both techniques. The extracellular staining technique was substantially quicker, and yielded similar frequencies of $CD4^{+}CD127^{+}CD25^{+}$ and $CD4^{+}CD127^{+}CD25^{+}$ Treg cell subsets, as compared to the conventional intra- and extracellular Treg staining methods. However, patients in this cohort also exhibited $FoxP3^{+}$ Treg cell subsets (Figure 5.4), which were missed when only the surface staining technique was employed.

5.3 Discussion

Despite efforts to improve cancer awareness in South Africa, patients suffering from breast and oesophageal cancer continue to present to healthcare centres with progressive disease. In this study, 88% of African women suffering from breast cancer were found to present to the healthcare centres with stage 3 and 4 disease, compared to only 12% of caucasian patients. This correlates well with findings by Vorobiof et al (2001) who assessed the different stages of disease presentation in 8411 women suffering from breast cancer in South Africa, and found that 77.7% of African women suffering from breast cancer presented with stage 3 and 4 disease, compared to 30.7% of non-African patients.

All the oesophageal cancer patients presented with stage 3 and 4 disease, rendering the majority of these participants eligible only for palliative treatment. Consequently, 60% of these oesophageal cancer participants demised within 3 months of presentation and 40% were lost to follow-up. This finding demonstrates that despite an 11 year lapse between this study and that conducted by Vorobiof et al (2010), racial disparities in health seeking behaviour has not improved and Black patients are still presenting with late stage disease. The delay by patients to seek conventional medical care has been attributed to a myriad of reasons including the patients' indigenous beliefs, fear of the stigma associated with a cancer diagnosis and mistrust of conventional treatment modalities (Vorobiof et al. 2001, American Cancer Society 2011). This finding illustrates that there is an urgent need to raise awareness regarding the morbidity and mortality associated with these conditions, the availability of treatment and the benefits of early presentation.

The extensive role which $CD4^+$ and $CD8^+$ T-lymphocytes play in the induction, regulation and mediation of antitumor immunity has already been described (Akhmetzyanova et al. 2013), and a high prevalence of intratumoral effector $CD4^+$ and $CD8^+$ T-cells has been associated with a better prognosis. T-lymphocytes express certain activation markers when stimulated, including $CD25^+$, $CD26^+$, $CD69^+$ $CD71^+$, and HLA-DR^+, depending on the stage of T-cell activation (Reddy et al. 2004, Starska et al. 2011). The early activation markers are regarded as $CD69^+$ and $CD71^+$, while $CD25^{high}$, $CD26^+$ and HLA-DR are regarded as markers of late activation (Starska et al. 2011). Thus the increased frequency of $CD25^+$ expressing $CD4^+$ and $CD8^+$ T-lymphocytes observed in this cohort may represent the immune systems response to the malignancy. These cell subsets could represent activated effector $CD4^+CD25^+$ and $CD8^+CD25^+$ T-cells, which are being recruited to the tumor site.

Although $CD25^+$ expression in the absence of FoxP3 and $CD127^{high}$ is representative of an active T-cell phenotype (Stanzani et al. 2004), it may also be associated with abnormal clonal expansion of antigen-specific T-lymphocytes (Starska et al. 2011, Nowicka et al. 2012). In this cohort, a large expansion in $CD25^+$ expressing $CD4^+$ and $CD8^+$ T-lymphocytes was observed, and although the small sample size prevented statistical analysis of some of the different groups, a statistically significant higher proportion of $CD4^+CD25^+$ T-lymphocytes was identified in patients presenting with stage 3 breast cancer (P=0.0039). This correlates with findings by Sasada and colleagues (2003) who reported significantly higher rates of $CD4^+CD25^+$ T-lymphocytes, ranging from 43.7- 86% of $CD4^+$ T-lymphocytes, in the peripheral blood of patients presenting with a variety of cancers including gastric, colorectal, pancreatic, oesophageal and liver carcinomas (Sasada et al. 2003). Contrary to Sasada and colleagues, we found a difference in the expression of $CD25^+$ T-lymphocytes in cancer patients at different stages of disease (Sasada et al. 2003). This could be because they only

included participants with gastrointestinal cancer, while the majority of this cohort consisted of breast cancer participants. This could suggest that $CD25^+$ expression may be tumor-specific; however, differences where observed in the frequency of $CD25^+$ expressing T-lymphocytes in both breast and oesophageal cancer participants in this cohort when stratified according to stage of disease (Figure 5.6 and Figure 5.7). Thus genetic and environmental factors would need to be investigated to identify which factors contribute to the differences observed in this cohort.

When stimulated in vitro $CD25^+$ expressing $CD4^+$ T-lymphocytes were shown to produce large quantities of Th2 cytokines, IL-4 and IL-10, with only small quantities of IL-2 and IFN-γ. Furthermore, $CD4^+CD25^+$ cells were shown to inhibit IL-2 and IFN-γ production by $CD4^+CD25^-$ T-lymphocytes (Sasada et al. 2003). The diversity associated with Treg cells makes it most probable that this population of cells could represent a $CD25^+CD127^-FoxP3^-$ Treg cell population, which has expanded secondary to the loss of immune surveillance. Liyanage and colleagues (2002) also observed a significantly higher prevalence of $CD4^+CD25^+$ T-lymphocytes in patients presenting with breast ($P<0.001$) and pancreatic cancer ($P<0.015$), as compared to normal controls. Although they did not analyse these cells for the expression of FoxP3 or CD127, they did find that these cell populations secreted large amounts of IL-10 and TGF-β, irrespective of whether they were isolated from cancer patients or normal controls (Liyanage et al. 2002). This could suggest that tumor-induced immune suppression may be mediated by the induction of $CD25^+$ expression and concurrent promotion of IL-10, IL-4 and TGF-β production by $CD4^+$ and $CD8^+$ T-lymphocyte. Further studies will need to be conducted to explore whether this population of $CD25^+CD127^-FoxP3^-$ cells express any additional markers of anergy or activation, as well as the functional suppressive activity and secreted cytokine profile of these cells.

Oesophageal cancer patients also showed an expansion in the $CD4^+CD25^+$ and $CD8^+CD25^+$ T-cell populations. Interestingly, patients presenting with stage 3 disease displayed higher frequencies of $CD4^+CD25^-$ and $CD8^+CD25^-$ T-lymphocytes as compared to patients presenting with stage 4 disease; however, significance was not achieved. Sasada and colleagues (2003) reported that $CD4^+CD25^-$ cells secreted IL-2 and IFN-γ when stimulated *in vitro*, which suggested a Th-1 like phenotype. This suggests that this cell population could represent an effector T-cell subset. Patients suffering from stage 4 disease generally present with substantial protein calorie malnutrition secondary to tumor-induced partial or total occlusion of the oesophagus, in conjunction with the metastatic spread of the disease. Therefore, the decline observed in the frequency of $CD4^+CD25^-$ effector T-lymphocytes in stage 4 patients as compared to stage 3 patients may be as a result of severe protein-energy malnutrition secondary to stage 4 oesophageal cancer, which has shown to induce immune paresis and decreases the frequency and functionality of T-lymphocyte subsets, with adverse consequences on the patient's prognosis (Haffejee et al. 1979). Furthermore, the increase in tumor burden and progression to a more advanced stage of disease could be as a direct response to a decline in $CD4^+CD25^-$ T-lymphocyte population.

Selective reduction in the number of $CD4^+CD25^-$ T-lymphocytes may also be attributed to their sensitivity to clonal deletion and apoptosis following induction by tumor derived factors, or $CD4^+CD25^+$ T-lymphocytes (Sasada et al. 2003).

Despite numerous studies reporting elevated levels of Treg cells in the peripheral blood of patients presenting with cancer (Liyanage et al. 2002, Wolf et al. 2003, Xu et al. 2009), this was not observed in our study (Wang et al. 2012). This may be as a consequence of our small samples size, or it could be due to insufficient and inadequate cellular markers which were

used in the identification of Treg cells in the majority of studies conducted previously (Wang et al. 2012). Breast and oesophageal cancer patients were shown to exhibit a variety of $CD4^+$ and $CD8^+$ Treg cell subpopulations, however $CD4^+FoxP3^+$ Treg cell levels were substantially lower than what was observed in a previously described South African control group (median 3.72% range 1.3%-7.5%) (Suchard et al. 2010). This correlates with findings by Szczepanik et al (2011), who also found lower rates of Treg cells in gastric cancer patients as compared to healthy control. This may suggest that conventional peripheral Treg cells are not elevated in the presence of cancer in this population or it may be that the current markers used in this study, including FoxP3, were not sufficient to identify Treg cells within the $CD4^+$ and $CD8^+$ T-cell populations.

Similar to Liu and colleagues (2006), we found that the extracellular staining technique of Treg cell subsets with $CD127^+$ and $CD25^+$ was efficient and substantially less time consuming, as compared to the more traditional intracellular (FoxP3) and extracellular Treg staining technique. However, this cohort was found to exhibit a wide variety of $FoxP3^+$ Treg cell subsets, including those which lacked CD127 expression. This suggests that the use of an extracellular staining technique would miss a substantial proportion of circulating Treg cells, which may have quantitative and qualitative implications in different disease types or different stages of disease (Liu et al. 2006).

Future work will include further characterisation of these T-cell subsets ($CD4^+CD25^+$, $CD8^+CD25^+$, $CD4^+CD25^-$ and $CD8^+CD25^-$ T-lymphocyte) and Treg cell subsets ($CD4^+CD25^+FoxP3^+$, $CD4^+CD127^+FoxP3^+$, $CD4^+CD25^+CD127^+$ and $CD8^+CD127^+FoxP3^+$, $CD8^+CD25^+CD127^+$ and $CD8^+CD25^+FoxP3^+$ Treg cell), the suppressive activity of each subset, and the role in immune response (e.g. cytokine production).

6 The evaluation of the relationship between $CD4^+$, $CD8^+$ and Treg cell populations and markers of prognosis in breast cancer: HER-2/neu and estrogen receptor status

6.1 Introduction

The quantification of hormonal and HER-2/neu receptor status in clinical practice has been shown as valuable prognostic and predictive markers in the treatment of breast cancer (Esteva et al. 2004, Weigelt et al. 2005). Over-expression of hormonal receptors has been associated with good prognosis in patients presenting with breast cancer, and predicts treatment response to hormonal therapy, including Tamoxifen treatment (Esteva et al. 2004). However, over-expression of the HER-2/neu oncogene has been associated with the development of more aggressive cancer with a poorer prognosis, higher recurrence rate and shorter survival time (Ciocca et al. 2006, Mitri et al. 2012). Recently, studies have demonstrated the role of HER-2/neu receptor as a predictor of treatment response to anti-Her-2/neu immunotherapy, including Trastuzumab (Ciocca et al. 2006). Numerous studies have demonstrated that an increase in the frequency of circulating or tumor associated T regulatory cells in patients presenting with breast cancer is also associated with poor prognosis (Nakamura, et al. 2009, Decker et al. 2012); however, other authors have disputed this claim (West et al. 2013). This chapter focuses on the association between the immune profile of patients presenting with breast cancer and HER-2/neu and oestrogen receptor status. Furthermore, the role of circulating Treg cells as a marker of prognosis was investigated. It was hypothesised that patients presenting with a “poor” prognostic receptor status would display elevated levels of circulating Treg cells as compared to patients presenting with a “good” prognostic receptor status.

6.2 Results

6.2.1 HER-2/neu, estrogen receptor status and association with stage of disease

The immunohistochemical staining for the hormonal and HER-2/neu receptor status of patients presenting with breast cancer at Kalafong hospital and Steve Biko Academic hospital was conducted by the National Health Laboratory Services of South Africa. The results of all patients included in this study were retrieved and analysed. The relationship between the stage of disease at presentation, oestrogen receptor, progesterone receptor and HER-2/neu receptor expression was evaluated.

Despite the fact that 64% of breast cancer patients presented with progressive disease (stage 3 and 4), the majority of patients presented with ER-positive (74% versus. 26%; P<0.0001), and HER-2/neu negative breast cancer (61% versus. 39%; P< 0.0001) (Figure 6.1). Only two patients were found to be both ER-negative and HER-2/neu-positive, which together indicates a much poorer prognosis than all other receptor combinations.

When patients were stratified according to the stage of disease at presentation, the majority of patients presenting with early breast cancer (stage 1 and 2) were found to express ER positivity (87%), compared to only 66% of patients presenting with progressive cancer (P=0.28) (Figure 6.2). Interestingly, similar levels of HER-2/neu expression were found in patients presenting with early (37.5%) and progressive breast cancer (40%) (P=0.63) (Figure 6.2).

As shown in figure 6.2, patients who presented with early disease (stage 1 and 2) were found to have a significantly higher prevalence of $ER^+/PR^+/HER\text{-}2/neu^-$ breast cancer, than patients with progressive disease (stage 3 and 4). In comparison to patients presenting with progressive disease, a significant proportion (50% versus. 6%, P=0.026) of patients presenting with early disease expressed (Figure 6.2). Only 12% of patients with early cancer as opposed to 20% of patients with progressive cancer presented with triple negative receptor status ($ER^-/PR^-/HER\text{-}2/neu^-$) (P=0.026) (Figure 6.2). While triple positive receptor status was observed in 13% of patients with early cancer, this was not observed in any of the patients presenting with progressive cancer (Figure 6.2). However, this finding did not reach statistical significance (P=0.405).

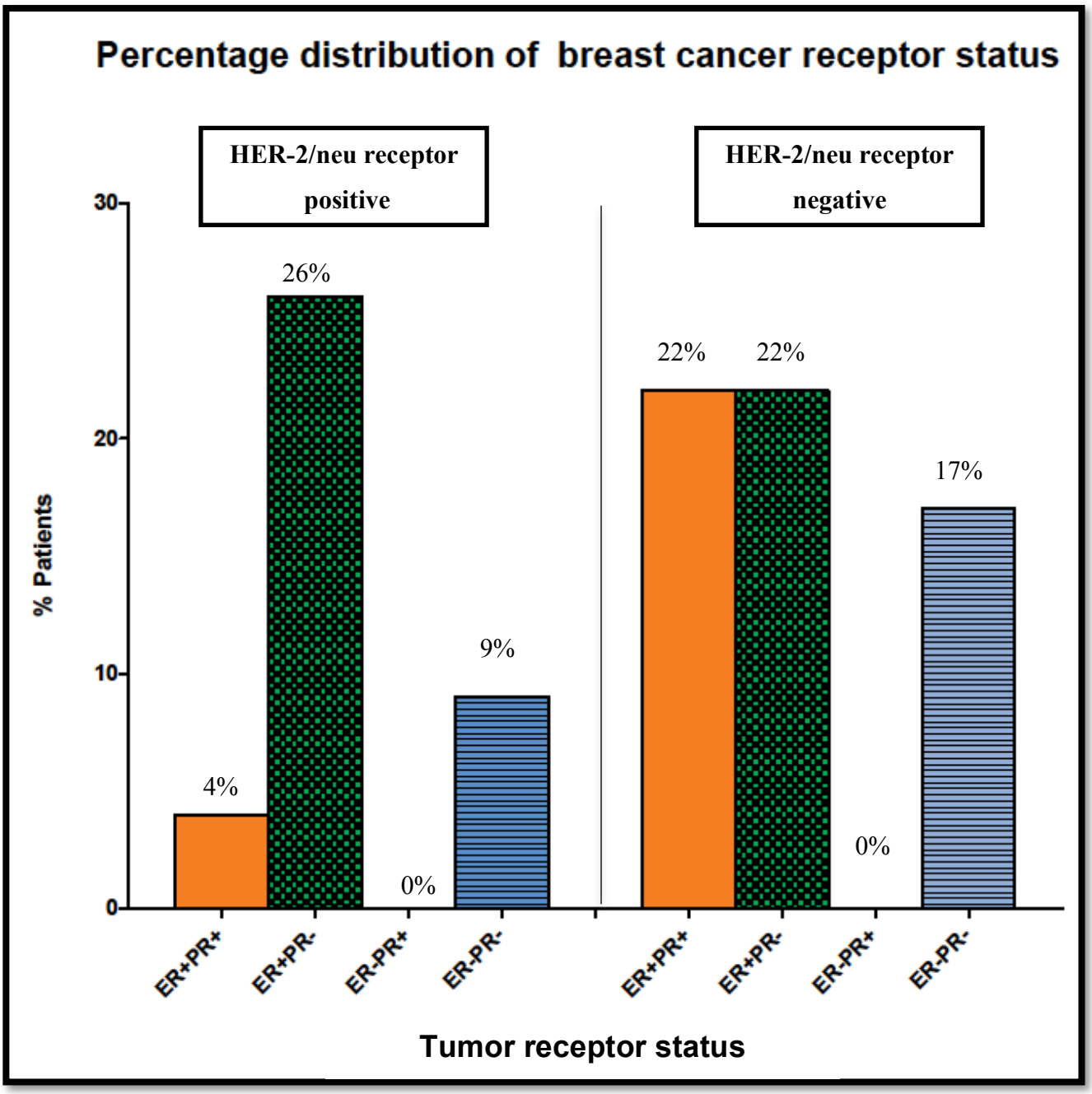


Figure 6.1: The percentage distribution of ER, PR and HER-2/neu receptor status in patients presenting with early and progressive breast cancer. A significantly higher proportion (61%) of breast cancer patients presented with HER-2/neu negative breast cancer, compared to HER-2/neu positive breast cancer (39%) (P<0.0001). A higher percentage of HER-2/neu negative women expressed ER-positivity, however this did not reach significance (P=0.078). The majority of women (74%) presented with ER^+ breast cancer (P<0.0001).

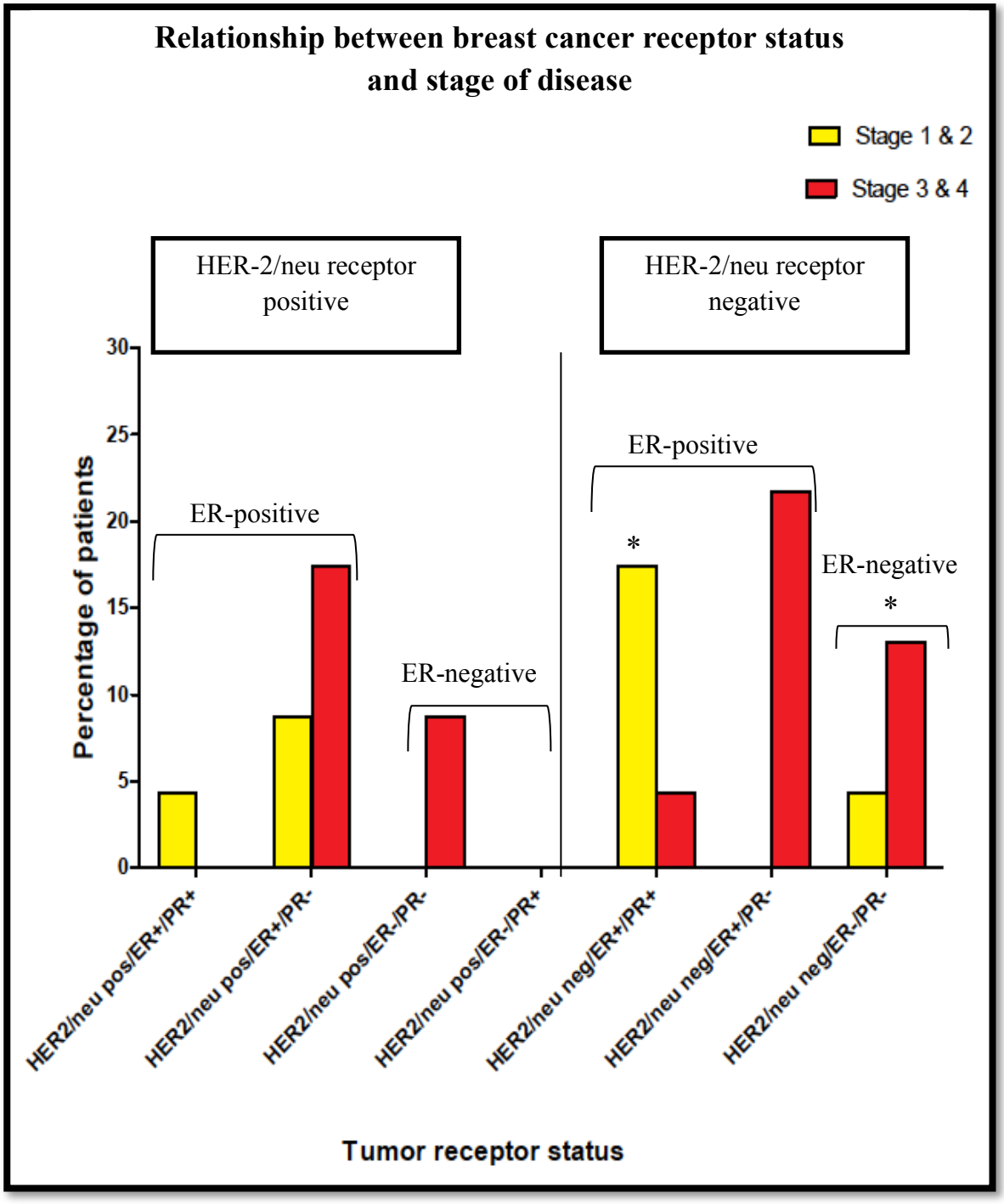


Figure 6.2: The relationship between receptor status and stage of disease, shown as a percentage distribution: 87% of patients presenting with stage 1and 2 cancer expressed ER-positive receptor, compared to 66% of patients presenting with progressive cancer. Triple positive status (HER2/neu pos/ER+/PR+) was only found in early cancer. (NS =not significant, *P<0.05, **P<0.01, ***P<0.001)

6.2.2 Evaluation of the immune profile, HER-2/neu and ER-receptor status

To determine whether the immune profile of breast cancer patients was associated with HER-2/neu and ER-receptor status, we compared the frequencies of non-Treg and Treg T-cell subsets in patients according to their tumor expression of HER-2/neu and ER-receptor. Furthermore, participants were stratified according to their stage of disease progression.

6.2.2.1 HER-2/neu receptor status

- *Non-Treg T-lymphocyte population*

Patients presenting with HER-2/neu positive breast cancer displayed a higher median $CD4^+$ count (388 cells/µl versus. 291 cells/µl) but lower median $CD8^+$ count (153 cells/µl versus. 171 cells/µl) as compared to patients presenting with HER-2/neu negative breast cancer (Table 6.1). These differences were not statistically significant (Table 6.1). HER-2/neu positivity was also associated with a significantly higher prevalence of circulating $CD8^+CD25^-$ T-lymphocytes (P=0.01) (Table 6.1).

When patients were stratified according to stage of disease at presentation, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher circulating $CD4^+$ count as compared to their HER-2/neu positive counterparts (P=0.05). Furthermore, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher $CD4^+$ count as compared to all patients presenting with late stage disease, irrespective of HER-2/neu status (P=0.05) (Table 6.2).

Table 6.1: Association between the general immune profile and HER-2/neu receptor status.

	HER-2/neu positive breast cancer		HER-2/neu negative breast cancer		
Cell type	**Median**	**IQR**	**Median**	**IQR**	
$CD4^+$ count (cells/µl)	388	134 - 554	291	213-473	P=0.80 (NS)
$CD8^+$ count (cells/µl)	153	56 - 263	171	102-230	P=0.87 (NS)
Total $CD4^+$%	50.05	37.95 – 52.24	50.31	42.6-56.66	P=0.75 (NS)
Total $CD8^+$ %	22.43	15.97-26.09	18.17	15.74-28.16	P=0.89 (NS)
$CD4^+CD25^+$ %	45.85	35.36-48.93	44.33	34.3-51.91	P=0.80 (NS)
$CD4^+CD25^-$%	4.15	2.30-6.75	2.00	0.73-4.44	P=0.10 (NS)
$CD8^+CD25^+$%	17.62	9.98-19.47	17.07	9.85-18.29	P=0.48 (NS)
$CD8^+CD25^-$%	4.76	1.35-8.40	1.10	0.47-2.39	P=0.01**
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)					

Patients presenting with early HER-2/neu negative breast cancer also displayed significantly higher $CD8^+$ T-lymphocyte counts as compared to early HER-2/neu positive cancer patients (P=0.05) (Table 6.2). A significantly higher percentage of $CD8^+CD25^-$ T-lymphocytes was observed in patients presenting with late HER-2/neu positive breast cancer, as compared to late HER-2/neu negative (P=0.04) and early HER-2/neu negative breast cancer (P=0.01) (Table 6.2).

Although the median percentage of the total $CD4^+$ and $CD4^+CD25^+$ T-lymphocytes observed in patients presenting with early HER-2/neu-negative breast cancer was increased as compared to patients presenting with HER-2/neu-positive early and progressive breast cancer; this was not statistically significant (Table 6.2).

No difference was observed in the remaining general immune T-cell populations.

Table 6.2: Association between the general immune profile and HER-2/neu receptor status, stratified according to the stage of disease.

Early cancer (Stage 1-2)					
	HER-2/neu positive breast cancer		**HER-2/neu negative breast cancer**		
Cell type	**Median**	**IQR**	**Median**	**IQR**	
$CD4^+$ count (cells/µl)	133	56-412	558	172-905	P=0.05*
$CD8^+$ count (cells/µl)	56	30-187	198	61-257	P= 0.05*
Total $CD4^+$%	48.49	47.91-50.65	56.16	48.41-60.39	P=0.10 (NS)
Total $CD8^+$ %	22.95	20.41-26.36	19.9	13.76-25.13	P=0.10 (NS)
$CD4^+CD25^+$ %	45.85	42.07-48.28	51.63	45.96-59.18	P=0.10 (NS)
$CD4^+CD25^-$%	2.4	2.36-6.79	2.06	0.66-11.23	P=0.18 (NS)
$CD8^+CD25^+$%	15.18	14.27-16.58	14.22	7.70-19.50	P=0.46 (NS)
$CD8^+CD25^-$%	4.01	0.56-8.63	0.69	0.17-3.42	P=0.17 (NS)
Late cancer (Stage 3-4)					
$CD4^+$ count (cells/µl	393	156-1133	259	154-341	P=0.19 (NS)
$CD8^+$count (cells/µl)	203	62-301	141	19-239	P=0.44 (NS)
Total $CD4^+$%	51.03	33.7-60.05	43.6	35.13-57.42	P=0.81 (NS)
Total $CD8^+$ %	21.23	14.33-26.41	21.86	8.39-32.43	P=0.47 (NS)
$CD4^+CD25^+$ %	44.63	33.53-56.80	37.42	26.27-47.05	P=0.24 (NS)
$CD4^+CD25^-$%	3.42	0.95-8.15	1.97	0.43-12.02	P=0.28 (NS)_
$CD8^+CD25^+$%	14.34	9.01-16.77	11.89	4.72-28.85	P=0.72 (NS)
$CD8^+CD25^-$%	5.89	1.42-9.59	1.81	0.53-3.1	P=0.04*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)					

- *Treg T-lymphocyte population*

No difference was observed in the circulating percentages of the different Treg cell subsets in patients presenting with early versus progressive breast cancer. When breast cancer patients were stratified according to their expression of HER-2/neu receptor status, patients presenting with early cancer displayed similar Treg profiles irrespective of HER-2/neu expression (Figure 6.3). However, patients presenting with progressive HER-2/neu negative cancer displayed a significantly higher frequency of $CD4^{+}CD127^{+}FoxP3^{+}$ Treg cells (P=0.036) (Figure 6.4), as compared to patients with progressive HER-2/neu positive breast cancer. $CD4^{+}CD25^{+}FoxP3^{+}$ (P=0.21) and $CD8^{+}CD25^{+}FoxP3^{+}$ (P=0.25) Treg levels in patients presenting with HER-2/neu negative cancer were also elevated in comparison to patients presenting with HER-2/neu positive breast cancer; however, this did not reach significance (Figure 6.4).

5.2.7 Intracellular versus extracellular staining

In order to explore the benefits of characterising and measuring circulating Treg cell subsets, with an extracellular staining technique, as opposed to the conventional combination of both intracellular and extracellular staining technique, blood samples from breast and oesophageal cancer patients were stained using both techniques. The extracellular staining technique was substantially quicker, and yielded similar frequencies of $CD4^{+}CD127^{+}CD25^{+}$ and $CD4^{+}CD127^{+}CD25^{+}$ Treg cell subsets, as compared to the conventional intra- and extracellular Treg staining methods. However, patients in this cohort also exhibited $FoxP3^{+}$ Treg cell subsets (Figure 5.4), which were missed when only the surface staining technique was employed.

5.3 Discussion

Despite efforts to improve cancer awareness in South Africa, patients suffering from breast and oesophageal cancer continue to present to healthcare centres with progressive disease. In this study, 88% of African women suffering from breast cancer were found to present to the healthcare centres with stage 3 and 4 disease, compared to only 12% of caucasian patients. This correlates well with findings by Vorobiof et al (2001) who assessed the different stages of disease presentation in 8411 women suffering from breast cancer in South Africa, and found that 77.7% of African women suffering from breast cancer presented with stage 3 and 4 disease, compared to 30.7% of non-African patients.

All the oesophageal cancer patients presented with stage 3 and 4 disease, rendering the majority of these participants eligible only for palliative treatment. Consequently, 60% of these oesophageal cancer participants demised within 3 months of presentation and 40% were lost to follow-up. This finding demonstrates that despite an 11 year lapse between this study and that conducted by Vorobiof et al (2010), racial disparities in health seeking behaviour has not improved and Black patients are still presenting with late stage disease. The delay by patients to seek conventional medical care has been attributed to a myriad of reasons including the patients' indigenous beliefs, fear of the stigma associated with a cancer diagnosis and mistrust of conventional treatment modalities (Vorobiof et al. 2001, American Cancer Society 2011). This finding illustrates that there is an urgent need to raise awareness regarding the morbidity and mortality associated with these conditions, the availability of treatment and the benefits of early presentation.

The extensive role which $CD4^+$ and $CD8^+$ T-lymphocytes play in the induction, regulation and mediation of antitumor immunity has already been described (Akhmetzyanova et al. 2013), and a high prevalence of intratumoral effector $CD4^+$ and $CD8^+$ T-cells has been associated with a better prognosis. T-lymphocytes express certain activation markers when stimulated, including $CD25^+$, $CD26^+$, $CD69^+$ $CD71^+$, and HLA-DR^+, depending on the stage of T-cell activation (Reddy et al. 2004, Starska et al. 2011). The early activation markers are regarded as $CD69^+$ and $CD71^+$, while $CD25^{high}$, $CD26^+$ and HLA-DR are regarded as markers of late activation (Starska et al. 2011). Thus the increased frequency of $CD25^+$ expressing $CD4^+$ and $CD8^+$ T-lymphocytes observed in this cohort may represent the immune systems response to the malignancy. These cell subsets could represent activated effector $CD4^+CD25^+$ and $CD8^+CD25^+$ T-cells, which are being recruited to the tumor site.

Although $CD25^+$ expression in the absence of FoxP3 and $CD127^{high}$ is representative of an active T-cell phenotype (Stanzani et al. 2004), it may also be associated with abnormal clonal expansion of antigen-specific T-lymphocytes (Starska et al. 2011, Nowicka et al. 2012). In this cohort, a large expansion in $CD25^+$ expressing $CD4^+$ and $CD8^+$ T-lymphocytes was observed, and although the small sample size prevented statistical analysis of some of the different groups, a statistically significant higher proportion of $CD4^+CD25^+$ T-lymphocytes was identified in patients presenting with stage 3 breast cancer (P=0.0039). This correlates with findings by Sasada and colleagues (2003) who reported significantly higher rates of $CD4^+CD25^+$ T-lymphocytes, ranging from 43.7- 86% of $CD4^+$ T-lymphocytes, in the peripheral blood of patients presenting with a variety of cancers including gastric, colorectal, pancreatic, oesophageal and liver carcinomas (Sasada et al. 2003). Contrary to Sasada and colleagues, we found a difference in the expression of $CD25^+$ T-lymphocytes in cancer patients at different stages of disease (Sasada et al. 2003). This could be because they only

included participants with gastrointestinal cancer, while the majority of this cohort consisted of breast cancer participants. This could suggest that $CD25^+$ expression may be tumor-specific; however, differences where observed in the frequency of $CD25^+$ expressing T-lymphocytes in both breast and oesophageal cancer participants in this cohort when stratified according to stage of disease (Figure 5.6 and Figure 5.7). Thus genetic and environmental factors would need to be investigated to identify which factors contribute to the differences observed in this cohort.

When stimulated in vitro $CD25^+$ expressing $CD4^+$ T-lymphocytes were shown to produce large quantities of Th2 cytokines, IL-4 and IL-10, with only small quantities of IL-2 and IFN-γ. Furthermore, $CD4^+CD25^+$ cells were shown to inhibit IL-2 and IFN-γ production by $CD4^+CD25^-$ T-lymphocytes (Sasada et al. 2003). The diversity associated with Treg cells makes it most probable that this population of cells could represent a $CD25^+CD127^-FoxP3^-$ Treg cell population, which has expanded secondary to the loss of immune surveillance. Liyanage and colleagues (2002) also observed a significantly higher prevalence of $CD4^+CD25^+$ T-lymphocytes in patients presenting with breast ($P<0.001$) and pancreatic cancer ($P<0.015$), as compared to normal controls. Although they did not analyse these cells for the expression of FoxP3 or CD127, they did find that these cell populations secreted large amounts of IL-10 and TGF-β, irrespective of whether they were isolated from cancer patients or normal controls (Liyanage et al. 2002). This could suggest that tumor-induced immune suppression may be mediated by the induction of $CD25^+$ expression and concurrent promotion of IL-10, IL-4 and TGF-β production by $CD4^+$ and $CD8^+$ T-lymphocyte. Further studies will need to be conducted to explore whether this population of $CD25^+CD127^-FoxP3^-$ cells express any additional markers of anergy or activation, as well as the functional suppressive activity and secreted cytokine profile of these cells.

Oesophageal cancer patients also showed an expansion in the $CD4^+CD25^+$ and $CD8^+CD25^+$ T-cell populations. Interestingly, patients presenting with stage 3 disease displayed higher frequencies of $CD4^+CD25^-$ and $CD8^+CD25^-$ T-lymphocytes as compared to patients presenting with stage 4 disease; however, significance was not achieved. Sasada and colleagues (2003) reported that $CD4^+CD25^-$ cells secreted IL-2 and IFN-γ when stimulated *in vitro*, which suggested a Th-1 like phenotype. This suggests that this cell population could represent an effector T-cell subset. Patients suffering from stage 4 disease generally present with substantial protein calorie malnutrition secondary to tumor-induced partial or total occlusion of the oesophagus, in conjunction with the metastatic spread of the disease. Therefore, the decline observed in the frequency of $CD4^+CD25^-$ effector T-lymphocytes in stage 4 patients as compared to stage 3 patients may be as a result of severe protein-energy malnutrition secondary to stage 4 oesophageal cancer, which has shown to induce immune paresis and decreases the frequency and functionality of T-lymphocyte subsets, with adverse consequences on the patient's prognosis (Haffejee et al. 1979). Furthermore, the increase in tumor burden and progression to a more advanced stage of disease could be as a direct response to a decline in $CD4^+CD25^-$ T-lymphocyte population.

Selective reduction in the number of $CD4^+CD25^-$ T-lymphocytes may also be attributed to their sensitivity to clonal deletion and apoptosis following induction by tumor derived factors, or $CD4^+CD25^+$ T-lymphocytes (Sasada et al. 2003).

Despite numerous studies reporting elevated levels of Treg cells in the peripheral blood of patients presenting with cancer (Liyanage et al. 2002, Wolf et al. 2003, Xu et al. 2009), this was not observed in our study (Wang et al. 2012). This may be as a consequence of our small samples size, or it could be due to insufficient and inadequate cellular markers which were

used in the identification of Treg cells in the majority of studies conducted previously (Wang et al. 2012). Breast and oesophageal cancer patients were shown to exhibit a variety of $CD4^+$ and $CD8^+$ Treg cell subpopulations, however $CD4^+FoxP3^+$ Treg cell levels were substantially lower than what was observed in a previously described South African control group (median 3.72% range 1.3%-7.5%) (Suchard et al. 2010). This correlates with findings by Szczepanik et al (2011), who also found lower rates of Treg cells in gastric cancer patients as compared to healthy control. This may suggest that conventional peripheral Treg cells are not elevated in the presence of cancer in this population or it may be that the current markers used in this study, including FoxP3, were not sufficient to identify Treg cells within the $CD4^+$ and $CD8^+$ T-cell populations.

Similar to Liu and colleagues (2006), we found that the extracellular staining technique of Treg cell subsets with $CD127^+$ and $CD25^+$ was efficient and substantially less time consuming, as compared to the more traditional intracellular (FoxP3) and extracellular Treg staining technique. However, this cohort was found to exhibit a wide variety of $FoxP3^+$ Treg cell subsets, including those which lacked CD127 expression. This suggests that the use of an extracellular staining technique would miss a substantial proportion of circulating Treg cells, which may have quantitative and qualitative implications in different disease types or different stages of disease (Liu et al. 2006).

Future work will include further characterisation of these T-cell subsets ($CD4^+CD25^+$, $CD8^+CD25^+$, $CD4^+CD25^-$ and $CD8^+CD25^-$ T-lymphocyte) and Treg cell subsets ($CD4^+CD25^+FoxP3^+$, $CD4^+CD127^+FoxP3^+$, $CD4^+CD25^+CD127^+$ and $CD8^+CD127^+FoxP3^+$, $CD8^+CD25^+CD127^+$ and $CD8^+CD25^+FoxP3^+$ Treg cell), the suppressive activity of each subset, and the role in immune response (e.g. cytokine production).

6 The evaluation of the relationship between $CD4^+$, $CD8^+$ and Treg cell populations and markers of prognosis in breast cancer: HER-2/neu and estrogen receptor status

6.1 Introduction

The quantification of hormonal and HER-2/neu receptor status in clinical practice has been shown as valuable prognostic and predictive markers in the treatment of breast cancer (Esteva et al. 2004, Weigelt et al. 2005). Over-expression of hormonal receptors has been associated with good prognosis in patients presenting with breast cancer, and predicts treatment response to hormonal therapy, including Tamoxifen treatment (Esteva et al. 2004). However, over-expression of the HER-2/neu oncogene has been associated with the development of more aggressive cancer with a poorer prognosis, higher recurrence rate and shorter survival time (Ciocca et al. 2006, Mitri et al. 2012). Recently, studies have demonstrated the role of HER-2/neu receptor as a predictor of treatment response to anti-Her-2/neu immunotherapy, including Trastuzumab (Ciocca et al. 2006). Numerous studies have demonstrated that an increase in the frequency of circulating or tumor associated T regulatory cells in patients presenting with breast cancer is also associated with poor prognosis (Nakamura, et al. 2009, Decker et al. 2012); however, other authors have disputed this claim (West et al. 2013). This chapter focuses on the association between the immune profile of patients presenting with breast cancer and HER-2/neu and oestrogen receptor status. Furthermore, the role of circulating Treg cells as a marker of prognosis was investigated. It was hypothesised that patients presenting with a “poor” prognostic receptor status would display elevated levels of circulating Treg cells as compared to patients presenting with a “good” prognostic receptor status.

6.2 Results

6.2.1 HER-2/neu, estrogen receptor status and association with stage of disease

The immunohistochemical staining for the hormonal and HER-2/neu receptor status of patients presenting with breast cancer at Kalafong hospital and Steve Biko Academic hospital was conducted by the National Health Laboratory Services of South Africa. The results of all patients included in this study were retrieved and analysed. The relationship between the stage of disease at presentation, oestrogen receptor, progesterone receptor and HER-2/neu receptor expression was evaluated.

Despite the fact that 64% of breast cancer patients presented with progressive disease (stage 3 and 4), the majority of patients presented with ER-positive (74% versus. 26%; P<0.0001), and HER-2/neu negative breast cancer (61% versus. 39%; P< 0.0001) (Figure 6.1). Only two patients were found to be both ER-negative and HER-2/neu-positive, which together indicates a much poorer prognosis than all other receptor combinations.

When patients were stratified according to the stage of disease at presentation, the majority of patients presenting with early breast cancer (stage 1 and 2) were found to express ER positivity (87%), compared to only 66% of patients presenting with progressive cancer (P=0.28) (Figure 6.2). Interestingly, similar levels of HER-2/neu expression were found in patients presenting with early (37.5%) and progressive breast cancer (40%) (P=0.63) (Figure 6.2).

As shown in figure 6.2, patients who presented with early disease (stage 1 and 2) were found to have a significantly higher prevalence of $ER^+/PR^+/HER\text{-}2/neu^-$ breast cancer, than patients with progressive disease (stage 3 and 4). In comparison to patients presenting with progressive disease, a significant proportion (50% versus. 6%, P=0.026) of patients presenting with early disease expressed (Figure 6.2). Only 12% of patients with early cancer as opposed to 20% of patients with progressive cancer presented with triple negative receptor status ($ER^-/PR^-/HER\text{-}2/neu^-$) (P=0.026) (Figure 6.2). While triple positive receptor status was observed in 13% of patients with early cancer, this was not observed in any of the patients presenting with progressive cancer (Figure 6.2). However, this finding did not reach statistical significance (P=0.405).

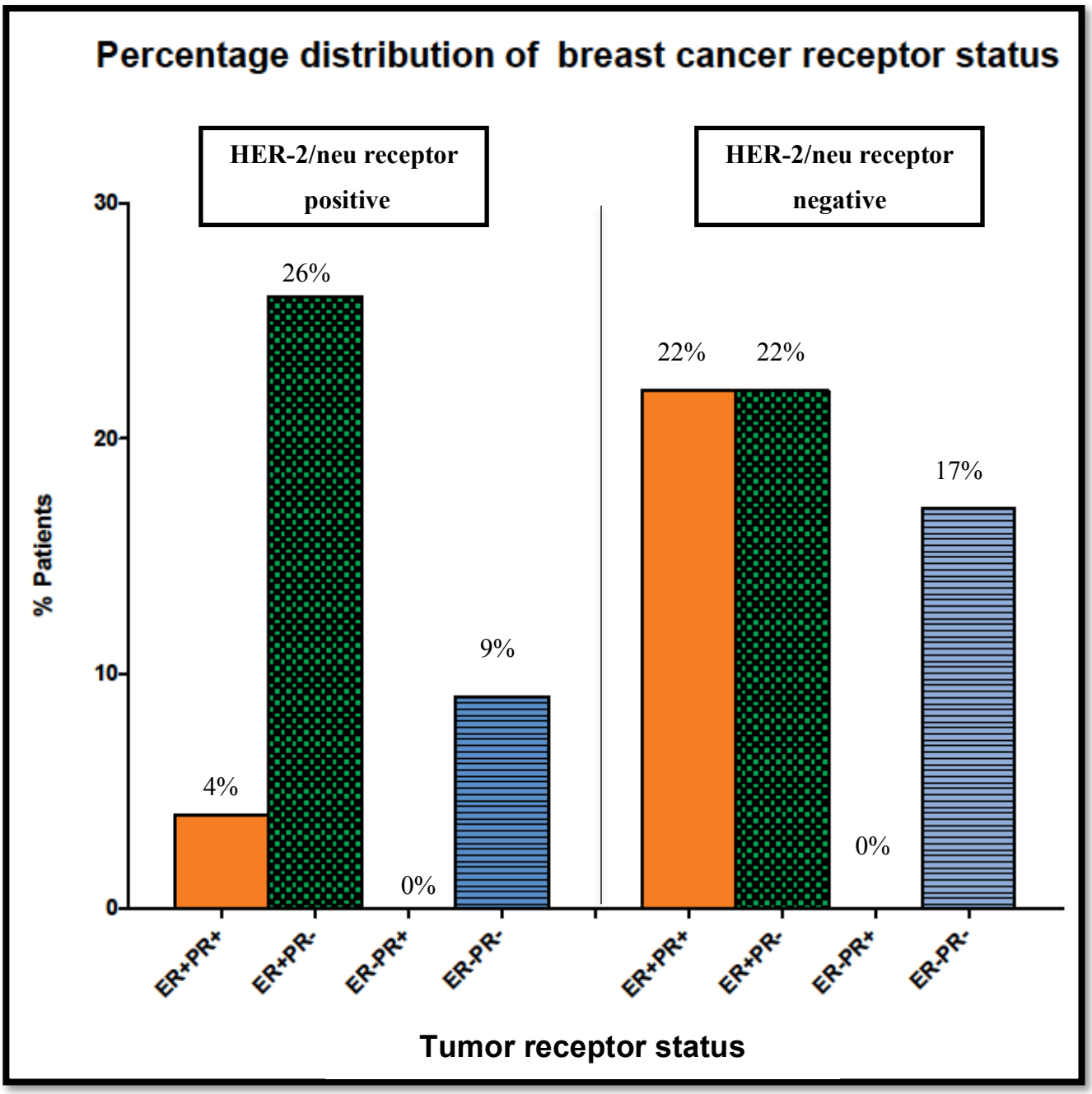


Figure 6.1: The percentage distribution of ER, PR and HER-2/neu receptor status in patients presenting with early and progressive breast cancer. A significantly higher proportion (61%) of breast cancer patients presented with HER-2/neu negative breast cancer, compared to HER-2/neu positive breast cancer (39%) (P<0.0001). A higher percentage of HER-2/neu negative women expressed ER-positivity, however this did not reach significance (P=0.078). The majority of women (74%) presented with ER^+ breast cancer (P<0.0001).

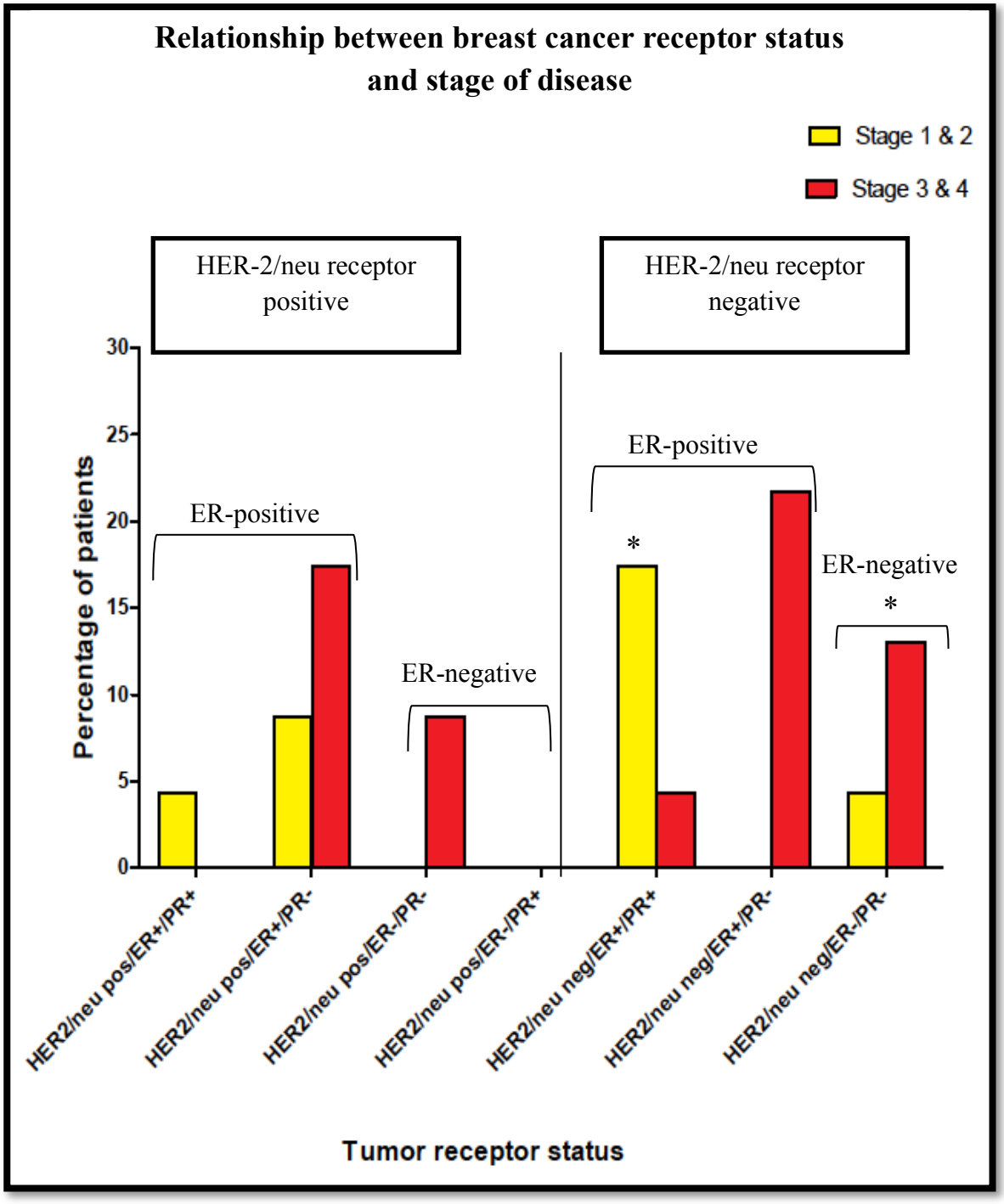


Figure 6.2: The relationship between receptor status and stage of disease, shown as a percentage distribution: 87% of patients presenting with stage 1and 2 cancer expressed ER-positive receptor, compared to 66% of patients presenting with progressive cancer. Triple positive status (HER2/neu pos/ER+/PR+) was only found in early cancer. (NS =not significant, *P<0.05, **P<0.01, ***P<0.001)

6.2.2 Evaluation of the immune profile, HER-2/neu and ER-receptor status

To determine whether the immune profile of breast cancer patients was associated with HER-2/neu and ER-receptor status, we compared the frequencies of non-Treg and Treg T-cell subsets in patients according to their tumor expression of HER-2/neu and ER-receptor. Furthermore, participants were stratified according to their stage of disease progression.

6.2.2.1 HER-2/neu receptor status

- *Non-Treg T-lymphocyte population*

Patients presenting with HER-2/neu positive breast cancer displayed a higher median $CD4^+$ count (388 cells/µl versus. 291 cells/µl) but lower median $CD8^+$ count (153 cells/µl versus. 171 cells/µl) as compared to patients presenting with HER-2/neu negative breast cancer (Table 6.1). These differences were not statistically significant (Table 6.1). HER-2/neu positivity was also associated with a significantly higher prevalence of circulating $CD8^+CD25^-$ T-lymphocytes (P=0.01) (Table 6.1).

When patients were stratified according to stage of disease at presentation, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher circulating $CD4^+$ count as compared to their HER-2/neu positive counterparts (P=0.05). Furthermore, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher $CD4^+$ count as compared to all patients presenting with late stage disease, irrespective of HER-2/neu status (P=0.05) (Table 6.2).

Table 6.1: Association between the general immune profile and HER-2/neu receptor status.

	HER-2/neu positive breast cancer		HER-2/neu negative breast cancer		
Cell type	**Median**	**IQR**	**Median**	**IQR**	
$CD4^+$ count (cells/µl)	388	134 - 554	291	213-473	P=0.80 (NS)
$CD8^+$ count (cells/µl)	153	56 - 263	171	102-230	P=0.87 (NS)
Total $CD4^+$%	50.05	37.95 – 52.24	50.31	42.6-56.66	P=0.75 (NS)
Total $CD8^+$ %	22.43	15.97-26.09	18.17	15.74-28.16	P=0.89 (NS)
$CD4^+CD25^+$ %	45.85	35.36-48.93	44.33	34.3-51.91	P=0.80 (NS)
$CD4^+CD25^-$%	4.15	2.30-6.75	2.00	0.73-4.44	P=0.10 (NS)
$CD8^+CD25^+$%	17.62	9.98-19.47	17.07	9.85-18.29	P=0.48 (NS)
$CD8^+CD25^-$%	4.76	1.35-8.40	1.10	0.47-2.39	P=0.01**
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)					

Patients presenting with early HER-2/neu negative breast cancer also displayed significantly higher $CD8^+$ T-lymphocyte counts as compared to early HER-2/neu positive cancer patients (P=0.05) (Table 6.2). A significantly higher percentage of $CD8^+CD25^-$ T-lymphocytes was observed in patients presenting with late HER-2/neu positive breast cancer, as compared to late HER-2/neu negative (P=0.04) and early HER-2/neu negative breast cancer (P=0.01) (Table 6.2).

Although the median percentage of the total $CD4^+$ and $CD4^+CD25^+$ T-lymphocytes observed in patients presenting with early HER-2/neu-negative breast cancer was increased as compared to patients presenting with HER-2/neu-positive early and progressive breast cancer; this was not statistically significant (Table 6.2).

No difference was observed in the remaining general immune T-cell populations.

Table 6.2: Association between the general immune profile and HER-2/neu receptor status, stratified according to the stage of disease.

Early cancer (Stage 1-2)					
	HER-2/neu positive breast cancer		**HER-2/neu negative breast cancer**		
Cell type	**Median**	**IQR**	**Median**	**IQR**	
$CD4^+$ count (cells/µl)	133	56-412	558	172-905	P=0.05*
$CD8^+$ count (cells/µl)	56	30-187	198	61-257	P= 0.05*
Total $CD4^+$%	48.49	47.91-50.65	56.16	48.41-60.39	P=0.10 (NS)
Total $CD8^+$ %	22.95	20.41-26.36	19.9	13.76-25.13	P=0.10 (NS)
$CD4^+CD25^+$ %	45.85	42.07-48.28	51.63	45.96-59.18	P=0.10 (NS)
$CD4^+CD25^-$%	2.4	2.36-6.79	2.06	0.66-11.23	P=0.18 (NS)
$CD8^+CD25^+$%	15.18	14.27-16.58	14.22	7.70-19.50	P=0.46 (NS)
$CD8^+CD25^-$%	4.01	0.56-8.63	0.69	0.17-3.42	P=0.17 (NS)
Late cancer (Stage 3-4)					
$CD4^+$ count (cells/µl	393	156-1133	259	154-341	P=0.19 (NS)
$CD8^+$count (cells/µl)	203	62-301	141	19-239	P=0.44 (NS)
Total $CD4^+$%	51.03	33.7-60.05	43.6	35.13-57.42	P=0.81 (NS)
Total $CD8^+$ %	21.23	14.33-26.41	21.86	8.39-32.43	P=0.47 (NS)
$CD4^+CD25^+$ %	44.63	33.53-56.80	37.42	26.27-47.05	P=0.24 (NS)
$CD4^+CD25^-$%	3.42	0.95-8.15	1.97	0.43-12.02	P=0.28 (NS)_
$CD8^+CD25^+$%	14.34	9.01-16.77	11.89	4.72-28.85	P=0.72 (NS)
$CD8^+CD25^-$%	5.89	1.42-9.59	1.81	0.53-3.1	P=0.04*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)					

- *Treg T-lymphocyte population*

No difference was observed in the circulating percentages of the different Treg cell subsets in patients presenting with early versus progressive breast cancer. When breast cancer patients were stratified according to their expression of HER-2/neu receptor status, patients presenting with early cancer displayed similar Treg profiles irrespective of HER-2/neu expression (Figure 6.3). However, patients presenting with progressive HER-2/neu negative cancer displayed a significantly higher frequency of $CD4^{+}CD127^{+}FoxP3^{+}$ Treg cells (P=0.036) (Figure 6.4), as compared to patients with progressive HER-2/neu positive breast cancer. $CD4^{+}CD25^{+}FoxP3^{+}$ (P=0.21) and $CD8^{+}CD25^{+}FoxP3^{+}$ (P=0.25) Treg levels in patients presenting with HER-2/neu negative cancer were also elevated in comparison to patients presenting with HER-2/neu positive breast cancer; however, this did not reach significance (Figure 6.4).

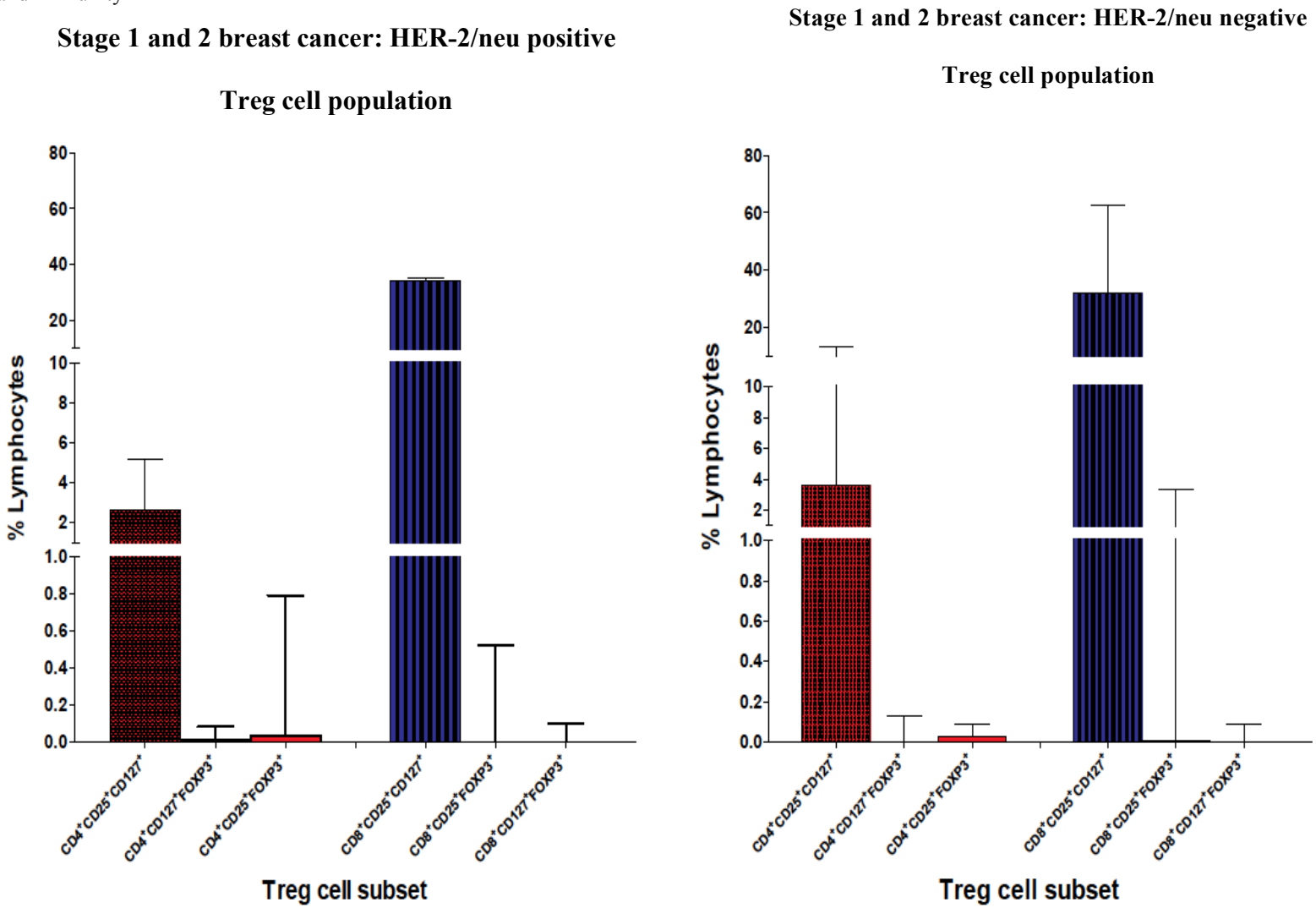
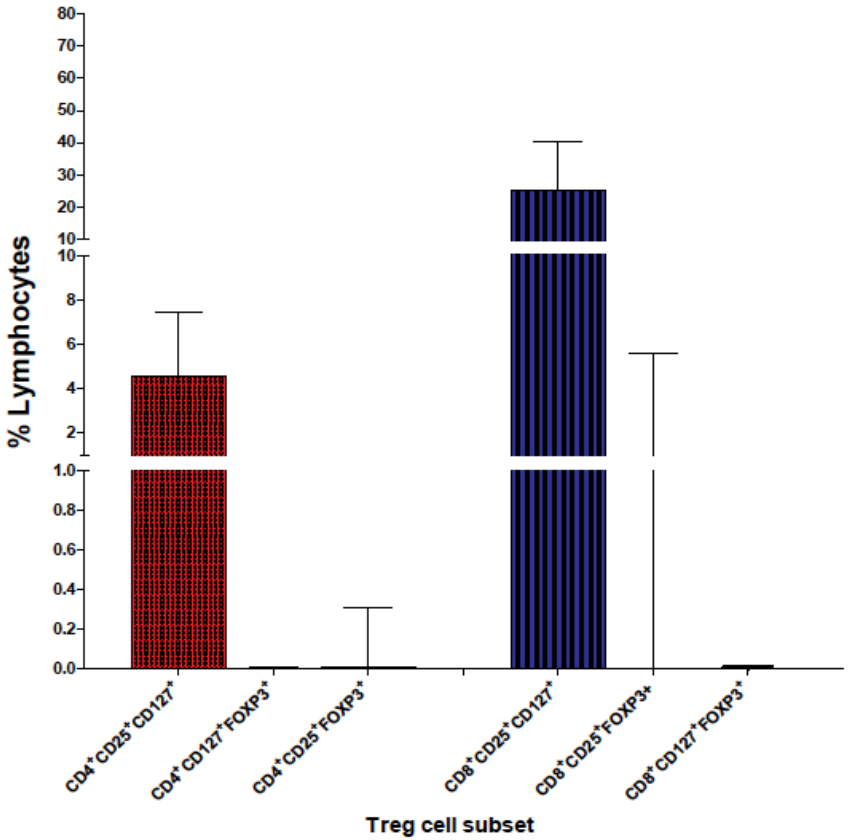


Figure 6.3: The percentage distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in patients presenting with stage 1 and 2 breast cancer, stratified according to HER-2/neu receptor status, shown as the median percentage ±IQR. No significant difference observed in the median lymphocyte percentage between patients presenting with HER-2/neu positive and HER-2/neu negative.
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)



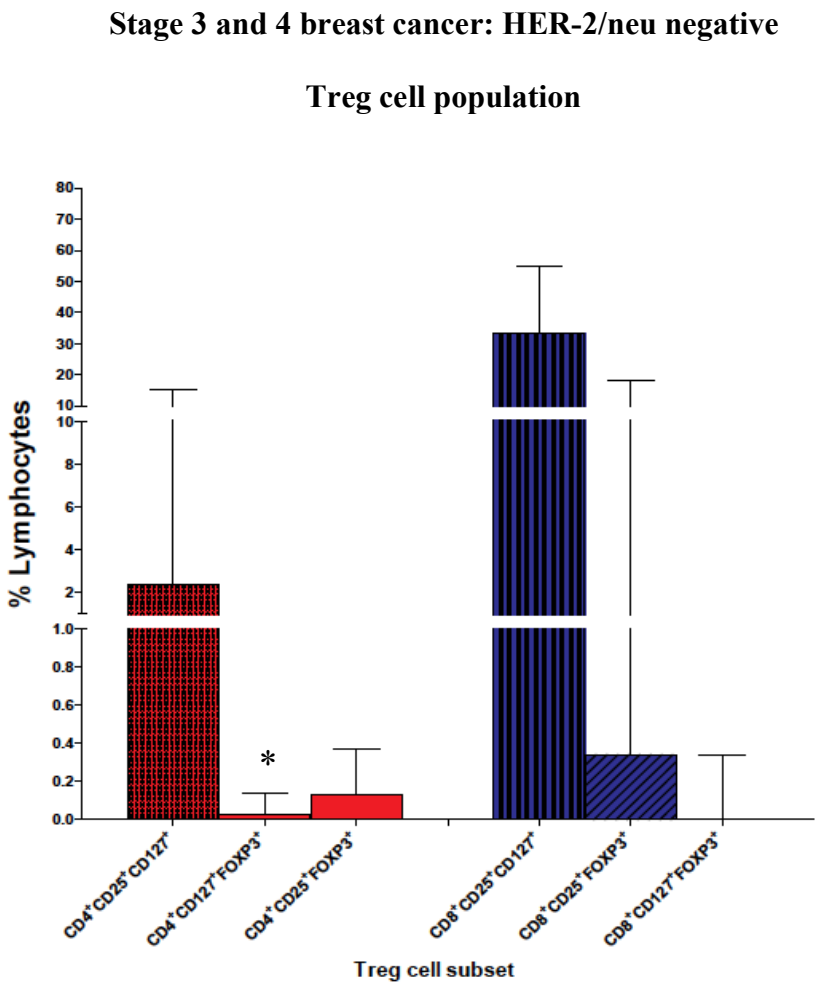


Figure 6.4: The percentage distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in patients presenting with stage 3 and 4 breast cancer, stratified according to HER-2/neu receptor status, shown as the median percentage ± IQR. Patients presenting with progressive HER-2/neu negative cancer displayed a significantly higher frequency of $CD4^+CD127^+FoxP3^+$ Treg cells (P=0.036) (Figure 6.4) as compared to patients presenting with HER-2/neu positive patients. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

6.2.2.2 Oestrogen receptor status

- *Non-Treg T-lymphocyte population*

Patients presenting with ER-positive and ER-negative breast cancer were found to have similar immune profiles at presentation (Table 6.3). However, in comparison to patients presenting with ER-positive cancer, patients presenting with ER-negative cancer were found to have a significantly elevated circulating percentage of $CD4^+CD25^+$ T-lymphocytes (42.71% versus. 53.33%; P=0.01). ER-positivity was also associated with an increased percentage of circulating total CD4% T-lymphocytes; however, this did not reach significance (56.33% versus. 48.49%; P=0.08) (Table 6.3). Surprisingly, although patients displaying ER-positivity are said to have a better prognosis, these patients demonstrated a lower $CD4^+$ and $CD8^+$ T-cell count as compared to patients presenting with ER-negative breast cancer (Table 6.3)

Table 6.3: Association between non-Treg immune profile and ER-receptor status

	ER-positive breast cancer		**ER-negative breast cancer**		
Cell type	**Median**	**IQR**	**Median**	**IQR**	
$CD4^+$ count (cells/µl)	303	172-425	312	132-1150	P=0.73 (NS)
$CD8^+$ count (cells/µl)	153	108-227	166	16-299	P=0.94 (NS)
Total $CD4^+$%	48.49	39.33-52.2	56.33	43.93-64.78	P=0.08 (NS)
Total $CD8^+$ %	22.43	20.03-26.35	15.89	8.01-31-60	P=0.12(NS)
$CD4^+CD25^+$ %	42.71	34.70-46.43	53.33	41.3-61.90	P=0.01 **
$CD4^+CD25^-$%	2.36	1.73-6.79	2.14	0.39-3.45	P=0.29 (NS)
$CD8^+CD25^+$%	14.27	11.07-15.64	11.93	4.72-30.23	P=0.44 (NS)
$CD8^+CD25^-$%	1.81	0.69-4.00	2.14	0.30-5.00	P=1.00 (NS)

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

When patients were stratified according to the stage of disease, ER-negativity, in comparison to ER-positivity, was still significantly associated with increased circulating levels of $CD4^+CD25^+$ T-lymphocytes (34.75 versus. 48.98; P=0.007) in patients presenting with progressive breast cancer. Furthermore, progressive ER-negative breast cancer was associated with increased circulating levels of total CD4% T-lymphocytes, however this also did not reach statistical significance (41.30-52.26; P=0.066). Only one patient presented with early ER-negative breast cancer, thus statistical analysis could not be performed.

Table 6.4: Association between non-Treg immune profile and ER-receptor status, stratified according to stage of disease

Early cancer (Stage 1-2)					
	ER positive breast cancer		**ER negative breast cancer**		
Cell type	**Median**	**95% Confidence interval**	**Median**	**IQR**	
$CD4^+$ count (cells/µl)	412	80-795	741	-	
$CD8^+$ count (cells/µl)	187	38-242	198	-	
Total $CD4^+$%	50.65	48.07-56.26	60.39	-	
Total $CD8^+$ %	20.41	15.70-25.97	16.14	-	
$CD4^+CD25^+$ %	46.69	43.26-52.81	59.18	-	
$CD4^+CD25^-$%	2.36	1.16-9.83	0.66	-	
$CD8^+CD25^+$%	15.18	8.76-18.58	14.22	-	
$CD8^+CD25^-$%	1.45	0.29-7.18	0.25	-	
Late cancer (Stage 3-4)					
$CD4^+$ count (cells/µl)	281	173-438	280	124-1196	P=0.80
$CD8^+$count (cells/µl)	153	108-260	135	12-306	P=0.62
Total $CD4^+$%	41.30	33.78-55.03	52.26	43.6-65.21	P=0.066
Total $CD8^+$ %	22.56	20.47-28.23	15.63	7.33-32.78	P=0.18
$CD4^+CD25^+$ %	34.75	27.96-42.85	48.98	40.62-62.2	P=0.007**
$CD4^+CD25^-$%	2.69	0.99-11.34	2.30	0.36-3.49	P=0.62
$CD8^+CD25^+$%	13.82	10.44-16.34	9.64	4.25-31.93	P=0.54
$CD8^+CD25^-$%	2.54	0.59-8.62	2.22	0.71-5.01	P=0.90
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)					

In addition to stratification according to ER-receptor status and stage of disease at presentation, patients were further stratified according to their HER-2/neu receptor status.

- **Early (Stage 1 and 2) ER-positive: HER-2/neu positive versus. HER-2/neu negative**

Patients presenting with early ER-receptor positive, HER-2/neu negative receptor status displayed a higher median $CD4^+$ count (492 cells/µl, 95% CI 172-905) as compared to patients presenting with early ER-receptor positive, HER-2/neu positive receptor status, (133 cells/µl, 95% CI= 56-412). However, this did not reach significance (P=0.077).

Similarly, patients with early ER-positive, HER-2/neu negative receptor status were also found to have higher median $CD8^+$ T-lymphocyte count (61 cells/µl, 95%CI 61-257) as compared to patients presenting with early ER-positive, HER-2/neu positive breast cancer (56 cells/µl, 95%CI 30-187). However, this difference did not reach statistical significance P=0.077), difference was observed in the remaining non-Treg immune markers (Figure 6.5).

- **Late (stage 3 and 4) HER-2/neu positive: ER-positive versus. ER-negative**

In comparison to patients presenting with progressive HER-2/neu positive, ER-positive breast cancer, patients presenting with progressive HER-2/neu positive, ER-negative breast cancer displayed an elevated median percentage of total $CD4^+$ T-lymphocytes (P=0.064) and $CD4^+CD25^+$ T-lymphocytes (P=0.064) (Figure 6.6). However, patients presenting with ER-positive, HER-2/neu positive breast cancer were found to have elevated levels of circulating total $CD8^+$ T-lymphocytes (P=0.064) and $CD8^+CD25^+$ T-lymphocytes (P=0.064) (Figure 6.6). These findings were not statistically significant.

- **Late (stage 3 and 4) HER-2/neu negative: ER-positive versus. ER-negative**

Patients presenting with late HER-2/neu negative, ER-negative breast cancer were found to have a significantly higher prevalence of circulating $CD4^{+}CD25^{+}$ T-lymphocytes, as compared to patients with ER-positive breast cancer (P=0.039) (Figure 6.7). Interestingly, patients who presented with ER-positive breast cancer displayed substantially higher levels of $CD4^{+}CD25^{-}$ Treg cells as compared to patients presenting with ER-negative breast cancer (Figure 6.7), however this did not reach significance.

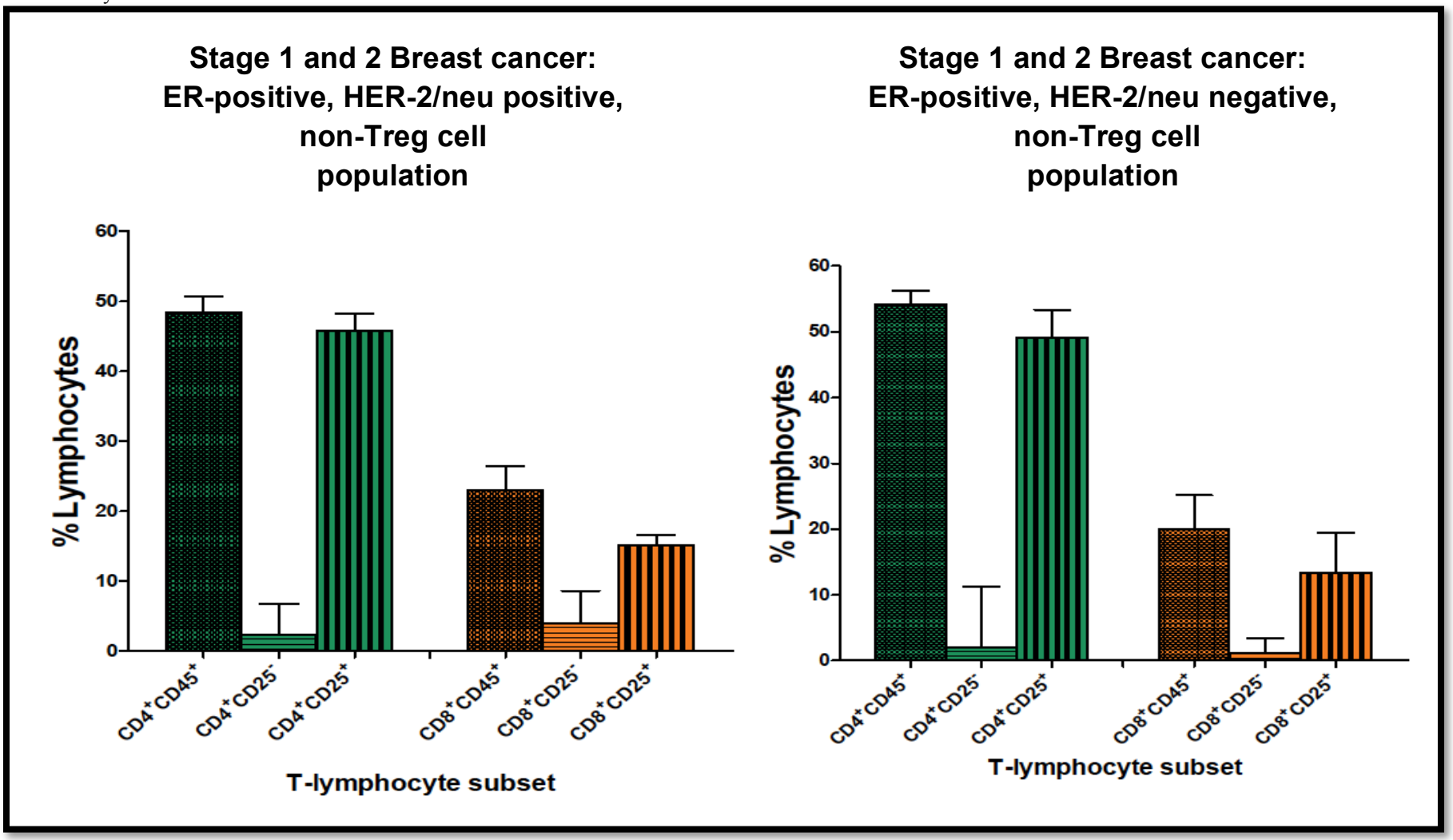


Figure 6.5: The $CD4^+$ and $CD8^+$ T-lymphocyte subsets in patients presenting with stage 1 and 2 breast cancer, stratified according to ER-receptor status, stage of disease and HER-2/neu receptor status, shown as the median % ±IQR. No difference was observed in the median non-Treg T-lymphocyte subsets in patients presenting with HER-2/neu positive and HER-2/neu negative patients. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

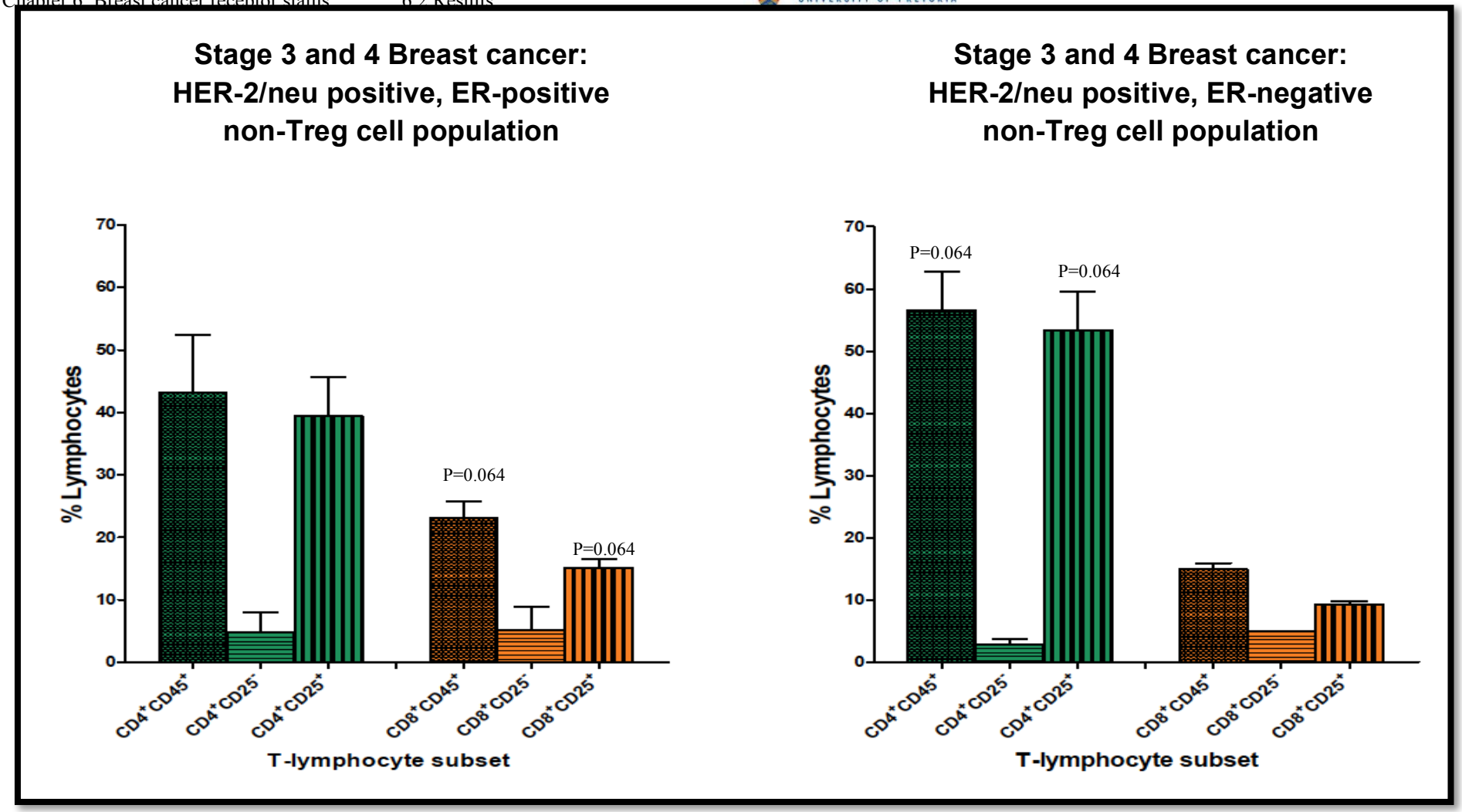


Figure 6.6: The $CD4^+$ and $CD8^+$ T-lymphocyte subsets in HER-2/neu positive breast cancer patients presenting with stage 3 and 4 disease, stratified according to ER-receptor status, and stage of disease and, shown as the median % ± IQR. The median $CD4^+CD45^+$ and $CD4^+CD25^+$ T-lymphocytes were elevated in patients presenting with ER-negative cancer compared to ER-positive cancer. The median $CD8^+CD45^+$ and $CD8^+CD25^+$ T-lymphocytes were elevated in patients presenting with ER-positive cancer, compared to ER-negative cancer, however, this did not reach significance. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

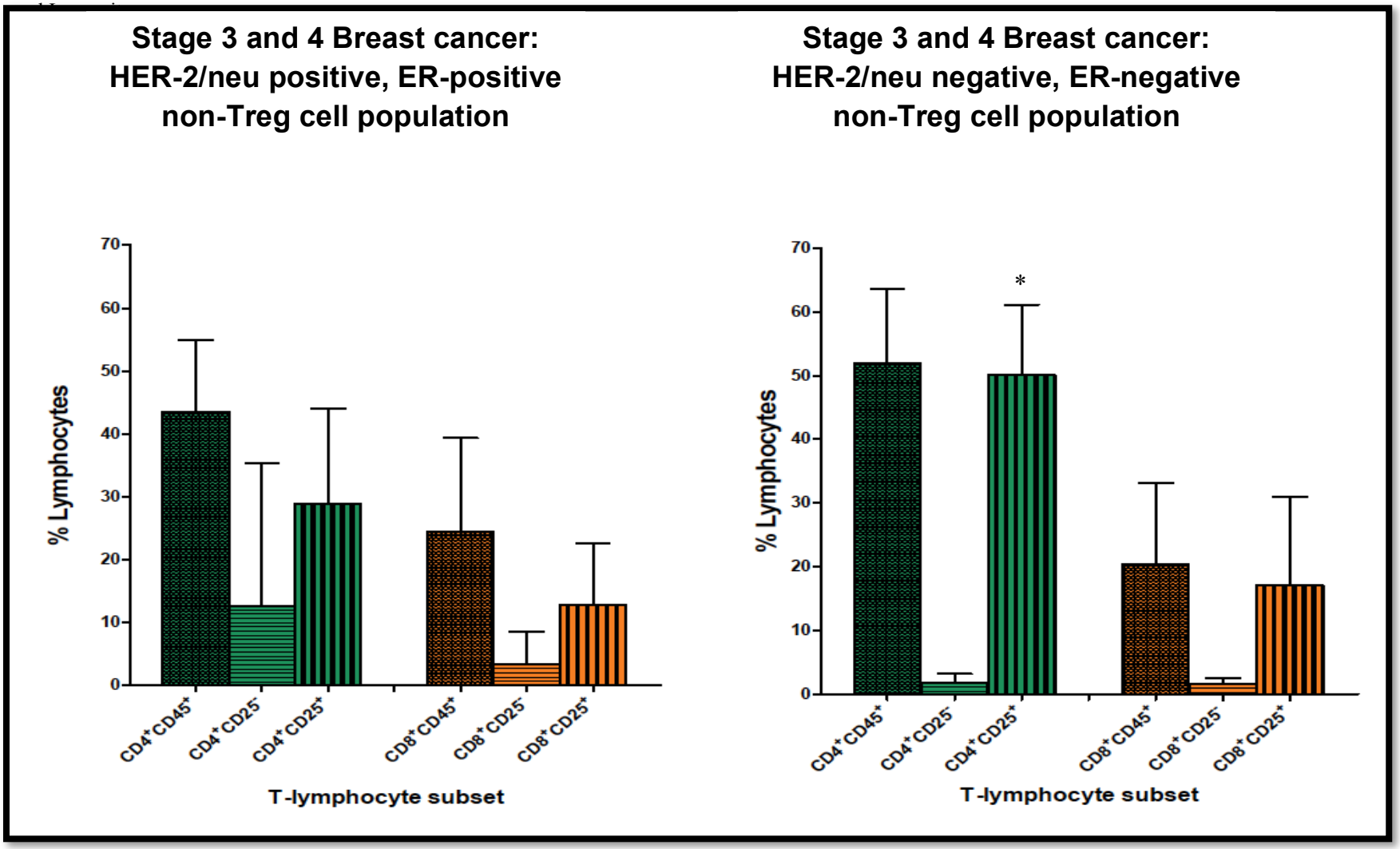


Figure 6.7: The $CD4^+$ and $CD8^+$ T-lymphocyte subsets in HER-2/neu negative breast cancer patients presenting with stage 3 and 4 disease, stratified according to ER-receptor status and stage of disease, shown as the median % ± IQR. The median $CD4^+CD25^+$ T-lymphocyte was found to be significantly elevated in patients presenting with ER-negative cancer as oppose to patients with ER-positive cancer (P=0.039) (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

- *Estrogen receptor status: Treg cell population*

A similar Treg cell profile was observed in patients presenting with early ER-positive, HER-2/neu positive breast cancer and early ER-positive, HER-2/neu negative breast cancer (Figure 6.8). Furthermore, no difference was observed in the Treg immune profile of patients presenting with progressive HER-2/neu positive breast cancer (ER-positive versus. ER-negative) (Figure 6.9) or HER-2/neu negative breast cancer (ER-positive versus ER-negative) (Figure 6.10).

The results of the association between the immune profile and the breast cancer tumor receptor status are summarised in table 6.5

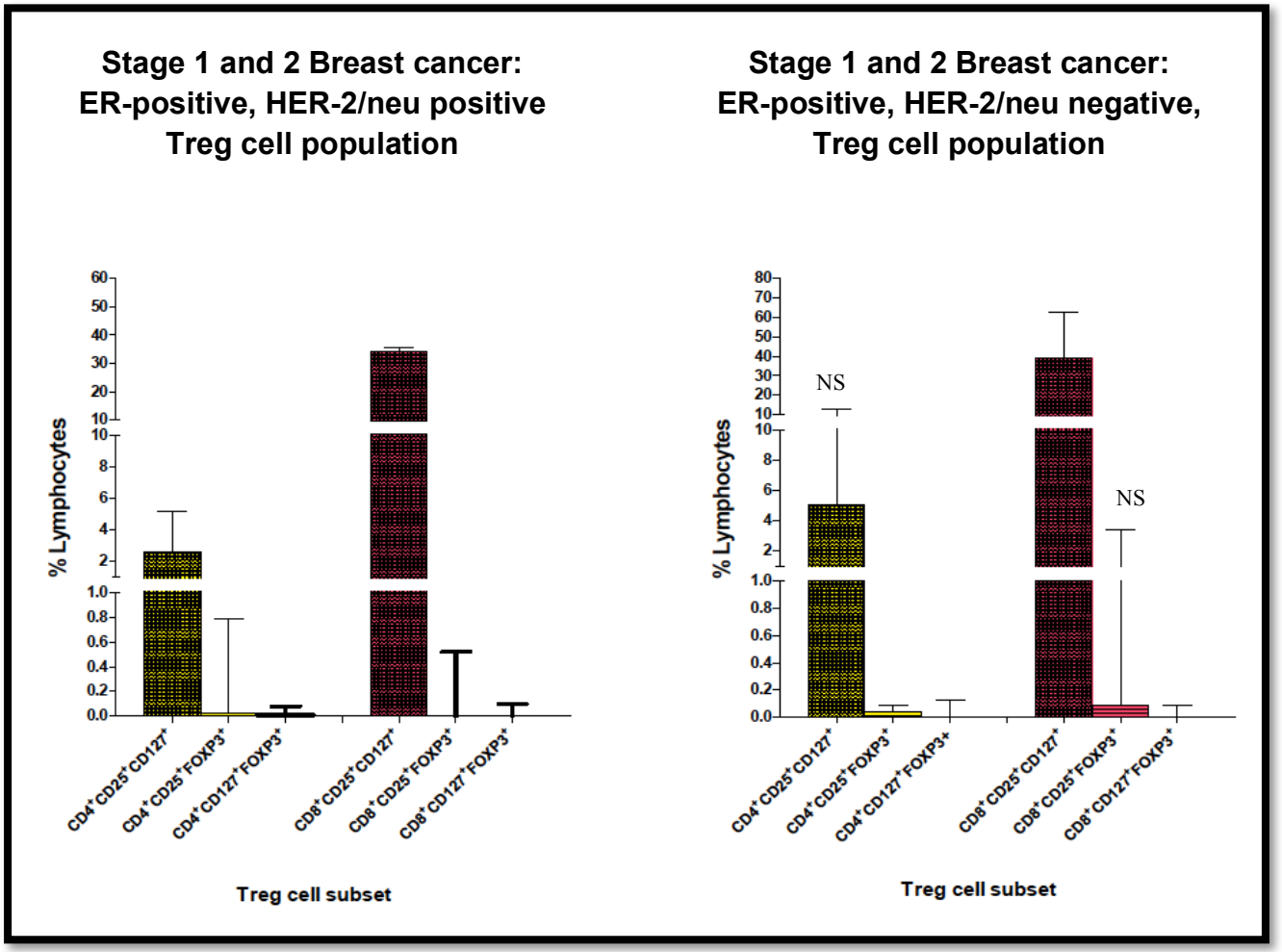


Figure 6.8: The $CD4^+$ and $CD8^+$ Treg subsets in patients presenting with ER-positive stage 1 and 2 breast cancer, stratified according to HER-2/neu receptor status, shown as the median % ±IQR. The median $CD4^+CD25^+CD127^+$ (P=0.4) and $CD8^+CD25^+FoxP3^+$ (P=0.6) T-lymphocytes were higher in patients presenting with HER-2/neu negative patients compared to HER-2/neu positive patients. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

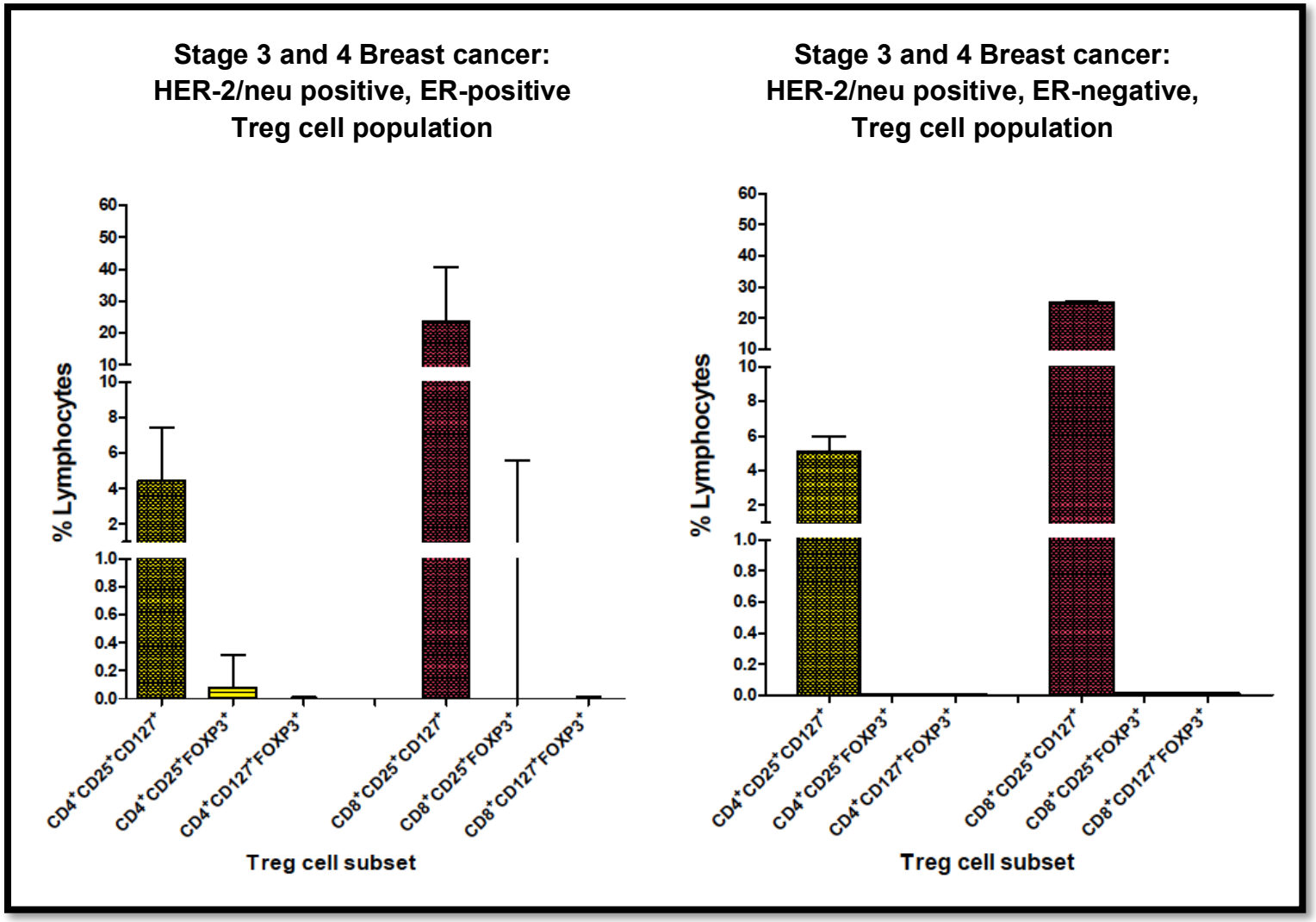


Figure 6.9: The $CD4^+$ and $CD8^+$ Treg subsets in patients presenting with HER-2/neu-positive stage 3 and 4 breast cancer, stratified according to ER-receptor status, shown as the median % ±IQR. No difference was observed in the median Treg cell subsets in ER-positive and ER-negative women.

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

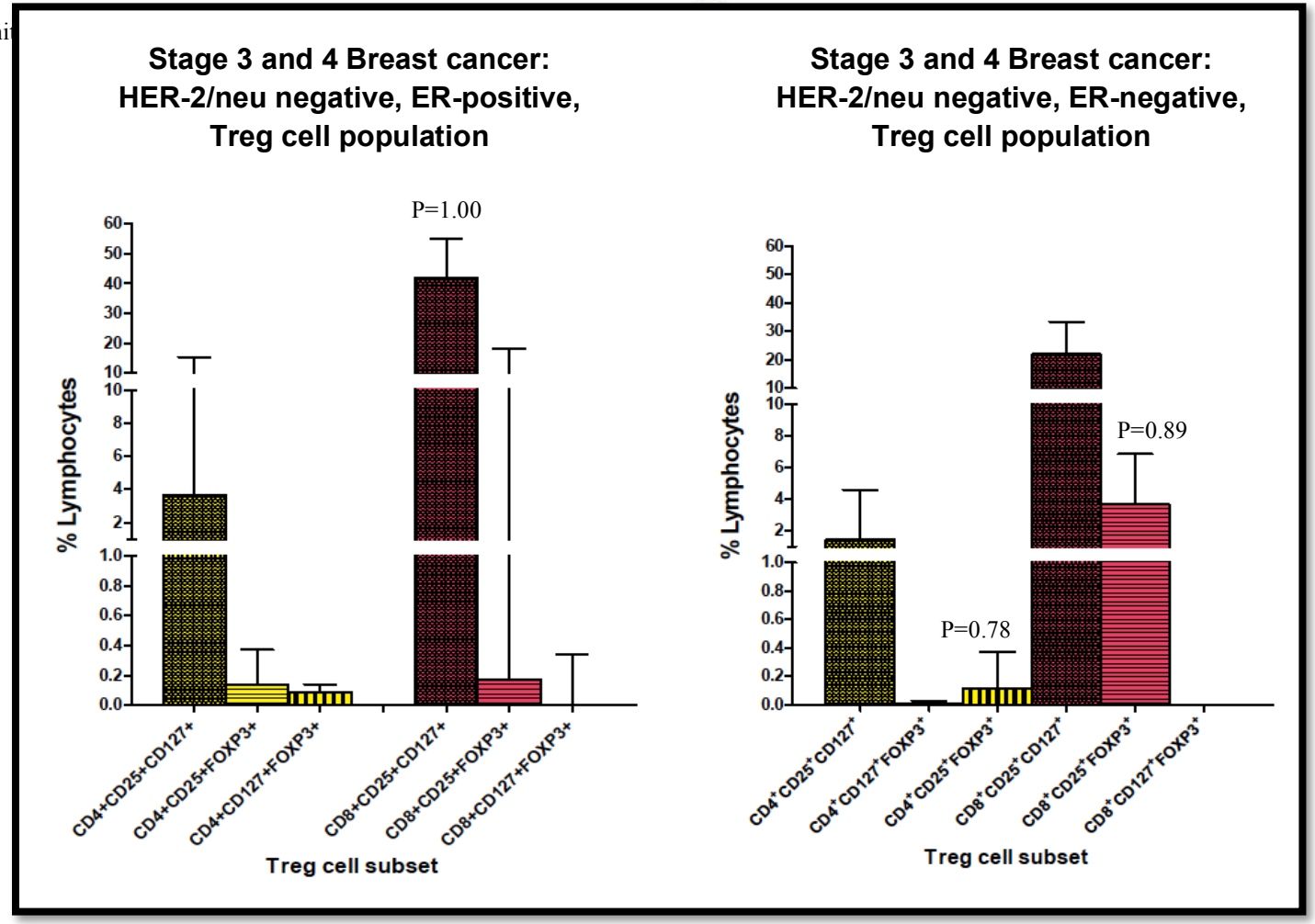


Figure 6.10: The $CD4^+$ and $CD8^+$ Treg subsets in patients presenting with HER-2/neu-negative stage 3 and 4 breast cancer, stratified according to ER-receptor status, shown as the median % ±IQR. The median $CD8^+CD25^+FoxP3^+$ and $CD4^+CD25^+FoxP3^+$Treg cell subsets were higher in patients presenting with ER-negative cancer compared to ER-positive cancer. The median $CD8^+CD25^+CD127^+$ Treg cells were higher in patients presenting with ER-positive, compared to ER-negative cancer.

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

Summary table 6.5: Association between immune profile and breast cancer tumor receptor status

			Non Treg T-cell population	Treg T-cell population
HER-2/neu receptor status	**HER-2/neu positive**	All breast cancers	High $CD4^+$ count (NS) Low $CD8^+$ count (NS) High $CD8^+CD25^-$ ** (£)	No difference observed
		Early breast cancer (Stage 1 and 2)	Low $CD4^+$ count* (§€) Low $CD8^+$ count* (¥) Low total CD4% (NS) Low $CD4^+CD25^+$ (NS)	No difference observed
		Late breast cancer (Stage 3 and 4)	Low CD4 count * (€) High $CD8^+CD25^-$ ** (₤) Low total CD4% (NS) Low $CD4^+CD25^+$ (NS)	Low $CD4^+CD127^+FoxP3^+$* ($) Low $CD8^+CD25^+FoxP3^+$ (NS) Low $CD4^+CD25^+FoxP3^+$ (NS)
	HER-2/neu negative	All breast cancers	Low CD4 count (NS) High CD8 count (NS) Low $CD8^+CD25^-$ ** (£)	No difference observed
		Early breast cancer (Stage 1 and 2)	High $CD4^+$ count * (§) High $CD8^+$ count * (¥) Low $CD8^+CD25^-$ ** (₤) High total CD4% (NS) High $CD4^+CD25^+$ (NS)	No difference observed
		Late breast cancer (Stage 3 and 4)	Low CD4 count* (€) Low $CD8^+CD25^-$ * (₤)	High $CD4^+CD127^+FoxP3^+$* ($) High $CD8^+CD25^+FoxP3^+$ (NS) High $CD4^+CD25^+FoxP3^+$ (NS)
ER-receptor status	**ER-positive**	All breast cancer	Low $CD4^+CD25^+$ ** (¶) High total CD4% (NS) Low $CD4^+$ count (NS) Low $CD8^+$ count (NS)	No difference observed
		Early breast cancer (Stage 1 and 2)	Unable to do statistics due to only 1 patient in the ER negative group.	Unable to do statistics due to only 1 patient in the ER negative group.
		Late breast cancer	Low $CD4^+CD25^+$ **(⌘)	No difference observed

		(Stage 3 and 4)	Low total $CD4^+$% (NS)	
	ER-negative	All breast cancer	High $CD4^+CD25^+$ ** (¶) Low total CD4% (NS) High $CD4^+$ count (NS) High $CD8^+$ count (NS)	No difference observed
		Early breast cancer (Stage 1 and 2)	Unable to do statistics due to only 1 patient in the group	Unable to do statistics due to only 1 patient in the group
		Progressive cancer (Stage 3 and 4)	High $CD4^+CD25^+$ ** (⌘) High total $CD4^+$% (NS)	No difference observed
Early ER-positive		HER-2/neu positive	Low $CD4^+$ count (NS) Low CD8 count (NS)	No difference observed
		HER-2/neu negative	High $CD4^+$ count (NS) High $CD8^+$ count (NS)	No difference observed
Late HER-2/neu positive		ER-positive	Low total $CD4^+$% (NS) Low $CD4^+CD25^+$% (NS) High total $CD8^+$% (NS) High $CD8^+CD25^+$ (NS)	No difference observed
		ER-negative	High total $CD4^+$% (NS) High $CD4^+CD25^+$% (NS) Low total $CD8^+$% (NS) Low $CD8^+CD25^+$ (NS)	No difference observed
Late HER-2/neu negative		ER-positive	Low $CD4^+CD25^+$ * (❖) High $CD4^+CD25^-$ (NS)	No difference observed
		ER-negative	High $CD4^+CD25^+$* (❖) Low $CD4^+CD25^-$ (NS)	No difference observed

NS =Not statistically significant, *= statistically significant: *P=0.05, **P=0.01, ***P>0.001

£= Statistically significance difference observed between patients presenting with HER-2/neu negative and HER-2/ neu positive breast cancer.

§ = Statistically significance difference observed between patients presenting with early HER-2/neu negative and early HER-2/ neu positive breast cancer.

€= Statistically significance difference observed between patients presenting with early HER-2/neu negative and late disease irrespective of HER-2/ neu receptor status.

¥= Statistically significance difference observed between patients presenting with early HER-2/neu negative and early HER-2/ neu positive breast cancer

₾= Statistically significance difference observed between patients presenting with late HER-2/neu positive and both early and HER-2/ neu negative breast cancer

$= Statistically significance difference observed between patients presenting with late HER-2/neu negative and HER-2/ neu positive breast cancer.
¶= Statistically significance difference observed between patients presenting with ER- negative and ER- positive breast cancer.
⌘ = Statistically significance difference observed between patients presenting with late ER- negative and ER- positive breast cancer
❖ = Statistically significance difference observed between patients presenting with late HER-2/neu negative ER- negative and HER-2/nu negative ER- positive breast cancer

6.3 Discussion

This chapter aimed to investigate the association between non-Treg and Treg immune profile, hormonal and HER-2/neu receptor status in patients presenting with early and progressive breast cancer.

The majority (74%) of patients in this cohort presented with ER-positive breast tumors, which is said to be associated with decreased rates of local invasion and distant metastases to other organs (Gago et al. 1998). This incidence is higher than that reported by other sub-Saharan African settings including, 43.2% by Yarney and colleagues (2008), in Ghana, and 24% by Bird and colleagues (2008) in Kenya (Yarney et al. 2008, Bird et al. 2008). Positive hormone receptor status has also been associated with improved overall survival and breast cancer-specific survival irrespective of additional clinically relevant factors (Zell et al. 2009). ER-negativity was only found in 12% of patients presenting with early disease, and 33% of patients presenting with progressive disease (P=0.28), and is a better determinant of poor survival rather than HER-2/neu status (Zell et al. 2009). In this cohort no mortalities occurred in patients presenting with ER-negative receptor status. However, of the patients who presented with ER-positive receptor status, 1 patient who presented with progressive HER-2/neu positive receptor status demised.

The over-expression of HER-2/neu receptor was found in 39% of breast cancer patients in this cohort, which was also higher than the 19% reported by Yarney and colleagues (2008) in a Ghanaian cohort, and 26% reported by Bogaert and colleagues (2013) in a rural South African cohort. HER-2/neu receptor expression is found in 25-30% of breast cancer, and develops during the late stages of carcinogenesis (Slamon et al. 1987, Gusterson et al. 1992, Ciocca et al. 2006). HER-2/neu positivity is said to give rise to aggressive tumors, which are

associated with shorter disease free periods and overall survival (Gusterson et al. 1992). HER-2/neu expression is said to be inversely correlated to ER-receptor status (Ciocca et al. 2006). However, despite our limited sample size, 31% of breast cancer patients over-expressed both HER-2/neu and oestrogen receptor, which is said to be uncommon, and has been associated with a poor overall survival (Ciocca et al. 2006). Co-expression is also associated with an up-regulation of HER-2/neu expression, which induces resistance to hormonal therapy e.g. Tamoxifen (Konecny et al. 2003). This data suggests that co-expression may not be as infrequent as previously suggested, and raises questions regarding the treatment options for this group of patients. Further studies need to be conducted on a larger sample size to evaluate the co-expression of these receptors in the South African population, and the impact that this status has on treatment outcome.

In contrast to findings by Bird and colleagues (2008), this cohort had lower levels of $ER^-/PR^-/HER\text{-}2/neu^+$ (17.6% versus. 8.7%), $ER^+/PR^+/HER\text{-}2/neu^+$ (8.8% versus. 4.3%) and triple negative ($ER^-/PR^-/HER\text{-}2/neu^-$) (44.1% versus.17.4%) breast cancer (Bird et al. 2008). On the other hand, we found a similar incidence of triple negative breast cancer to that reported by Cubasch and colleague (2013) in a different South African setting (19.1% versus. 17.4%) (Cubasch et al. 2013). This may suggest that genetic or even environmental factors may play a role in the differences observed in receptor status in patients in the West African cohort, versus those found in this setting. However, irrespective of the location, this subgroup is a cause for concern considering the fact that triple negative status has been associated with more advanced histological grade, higher recurrence rate, a decreased overall survival and substantial hormonal insensitivity (Rakha et al. 2007). This was substantiated by this cohort, where early disease was significantly associated with ER^+/PR^+HER-2/neu negative status

(P=0.026), while progressive disease was significantly associated with triple negative status ($ER^-/PR^-/HER\text{-}2/neu^-$) (P=0.026).

Patients presenting with early HER-2/neu negative cancer were found to have a higher circulating $CD4^+$ and $CD8^+$ count, as compared to patients presenting with early HER-2/neu positive disease or late disease. This may suggest that a positive HER-2/neu receptor status may be associated with a poorer immune profile, and possibly poorer antitumor effector activity. This could potentially contribute to the poor survival outcome which has been atributed to this marker.

Patients presenting with early HER-2/neu positive breast cancer displayed a higher frequency of circulating $CD8^+CD25^-$ T-lymphocytes (P=0.01) as compared to patients presenting with HER-2/neu negative early (P=0.01) and late breast cancer (P=0.04). As previously discussed (Chapter 5), $CD8^+CD25^-$ T-lymphocyte is not clearly characterised in the literature but could represent an effector T-lymphocyte population.

ER-receptor status alone did not seem to have an impact on the immune profile; however, when assessed in conjunction with the stage of disease at presentation, patients presenting with progressive ER-negative cancer displayed a significantly higher prevalence of $CD4^+CD25^+$ T-lymphocytes (P=0.007), as compared to patients presenting with progressive ER-positive cancer. As mentioned previously (chapter 5), this T-cell population could potentially represent a suppressive T-cell subset. As the remaining non-Treg cell subsets were similar between the groups, this suggests that ER-negativity may be associated with an up-regulation of suppressive T-cell activity, and suggests that these cells may be involved in

mediating a suppressive and tolerant immune milieu that facilitates tumor progression. This suggests that ER-receptor status may serve as an indicator of the patients' immune status, with ER-negativity possibly being associated with a poorer immune status.

Early ER-positive, HER-2/neu-negative breast cancer were found to have a higher $CD4^+$ count (492 cells/µl-133 cells/µl, P=0.07), and $CD8^+$ count (61 cells/µl-56 cells/µl; P=0.07), (Figure 6.5) as compared to patients presenting with early ER-receptor positive, HER-2/neu positive receptor status and all patients with progressive breast cancer. Although this was not statistically significant, within the clinical setting it may suggest that $CD4^+$ and $CD8^+$ T-cell immunity in this group of patients is still intact, compared to patients with more progressive disease. This suggests that the role of $CD4^+$ and $CD8^+$ T-lymphocytes in the induction and maintenance of antitumor immunity is sustained in this group of patients. Furthermore, this suggests that HER-2/neu status may also reflect the immune status of a patient, with HER-2/neu over-expression possibly being associated with a poorer immune status.

Interestingly, a variety of $CD4^+$ and $CD8^+$ Treg cell populations were also identified in this cohort, with a trend towards higher frequencies of $CD8^+CD25^+CD127^+$, $CD8^+CD25^+FoxP3^+$ and $CD4^+CD25^+CD127^+$ Treg cells being detected in patients diagnosed with early cancer compared to late cancer, though significance was not reached. However, we did not observe an increased prevalence of Treg cells in the peripheral blood of patients presenting with HER-2/neu positive cancers as was reported by Perez & colleagues (2007) and Rech and colleagues (2010). In fact, the majority of Treg cell subsets were identified in patients presenting with progressive HER-2/neu negative cancer (Figure 6.10). Furthermore, patients presenting with late HER-2/neu positive receptor status (Figure 6.9) demonstrated similar

Treg immune profiles to patients presenting with early ER-positive receptor status (Figure 8). This suggests that an increase in Treg cell subset is not associated with the clinical stage of disease or the ER-receptor status. It may suggest that within our setting the presence of Treg cell subsets is associated with good HER-2/neu receptor status (HER-2/neu negative).

Furthermore, as previously mentioned, early HER-2/neu negative receptor status was also associated with an increase in non-Treg immune profile as compared to early HER-2/neu positive receptor status, which may suggest that HER-2/neu receptor status may serve as a fair representation of a patients' immune status and vice-versa. Further studies with a larger sample size will need to be conducted to confirm or refute this finding.

To the best of my knowledge, this is the first study to compare biological immune profiles with hormonal and HER-2/neu receptor status in African patients presenting with breast cancer. Furthermore, this is the first study to demonstrate circulating $CD4^+$ and CD8 Treg cell populations in African patients presenting with breast cancer. Although I have found a number of contradictory findings as compared to what has been previously reported, this could be as a result of the small sample size or due to the fact that no other study has assessed the prevalence of Treg cell populations in the presence of breast cancer within an African population. Further studies need to be conducted with a larger sample size to investigate this further.

7 Effect of antitumor treatment on T-lymphocyte distribution

7.1 Introduction

Research efforts focused on the augmentation of antitumor immunity through the manipulation of cellular and soluble components of the immune system has been on going for more than 100 years (Agarwala et al. 2010). These efforts have been intensified following the discovery of Treg cells, with numerous studies attempting to facilitate immune surveillance through the depletion of circulating and intratumoral Treg cell populations in cancer patients (Tuve et al. 2007). This mode of treatment would be used as an adjunct to conventional antitumor treatment (surgery, chemotherapy and radiotherapy) and would result in the amplification of the hosts antitumor immunity while simultaneously attenuating the profound immunosuppressive effects associated with orthodox antitumor treatment (Lennard et al. 1985, Baumgartner et al. 2009).

Surgery, chemotherapy and radiotherapy have all been shown to induce defects in T-lymphocyte proliferation, function and cytokine secretion, with a consequent reduction in the circulating T-lymphocyte population (Campbell et al. 1973, Rasmussen et al. 1982, Lennard et al. 1985, De-en et al. 1988, Hensler et al. 1997, González et al. 2007). This creates an environment that is conducive to tumor dissemination, and is associated with an increased risk of relapse following treatment (De-en et al. 1988, Kachikwu et al. 2011, Kang et al. 2009). However, conventional antitumor treatment has also been shown to facilitate antitumor immunity through the depletion of Treg cells. This occurs either following the surgical removal of the tumor mass, which contains a large proportion of the Treg cells, or via apoptosis of Treg cells following exposure to chemotherapeutic agents or radiation (Liu et al. 2012, Schmidt et al. 2012, Wang et al. 2013, Baumgartner et al. 2008). Therefore, in

theory conventional treatment should diminish the Treg mediated immunosuppressive effect and promote antitumor immunity.

Despite numerous reports on the effect which cancer treatment has on cells involved in antitumor immunity ($CD4^+$ and $CD8^+$ T-cells) (De-en et al. 1998, Ishikawa et al. 2009, Kang et al. 2009) and Treg cell populations (Ladoire et al. 2008, Kachiwu et al. 2011, Schmidt et al. 2012), little is known about the overall effect of antitumor treatment on the relationship between T-cells which promote tumor progression (Treg cells) and those which promote tumor regression (non-Treg $CD4^+$ and $CD8^+$ T-cells). We aimed to investigate whether the overall effect of antitumor treatment creates an environment which is more conducive to tumor growth (increased Treg and decreased non-Treg cells) or tumor eradication (decreased Treg and increased non-Treg cells).

In order to evaluate the effect of conventional antitumor treatment, including surgery, chemotherapy and radiotherapy, on T-lymphocyte subsets in patients presenting with breast and oesophageal cancer, peripheral blood samples were collected from the treatment naïve patients, to establish a baseline T-lymphocyte profile, and at day 1 post antitumor treatment. An additional blood sample was collected at 5-7 weeks post-treatment to determine whether any change observed at day 1 post-treatment had returned to baseline levels. The 5-7 week time frame was chosen because most patients who received surgical intervention were started on adjuvant chemotherapy no later than 5-7 weeks post-surgery.

Twenty-five breast cancer and 10 oesophageal cancer patients supplied blood samples for examination of baseline T-lymphocyte profile. At day 1 post-treatment only 24 breast and oesophageal cancer patients in total were able to supply blood samples for post-treatment examination. At the 5-7 week follow-up only 13 cancer patients from the original 35 were able to supply blood samples for examination. The reasons for patients not supplying samples included death, early start on 2nd line treatment and loss to follow-up.

7.2 Results

7.2.1 Breast and oesophageal cancer: All antitumor treatment

7.2.1.1 Detection of variation in circulating non-Treg $CD4^+$ and $CD8^+$ T-lymphocyte percentage at day 1 post-treatment and at 5-7 weeks post-treatment, in comparison to baseline levels

In order to assess the effect of antitumor treatment on T-lymphocyte immune profile, blood samples were taken at baseline (before treatment), day 1 post-treatment and at 5-7 weeks post-treatment. All $CD4^+$ T-cell populations showed a non-significant decline in the median T-cell percentages at 5-7 weeks post-treatment as compared to baseline levels (Figure 7.1). An analysis of variance (Kruskal-Wallis) revealed a non-significant variation in the median percentage of total $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T-lymphocytes at day 1 post-treatment and 5-7 weeks post-treatment, as compared to baseline T-lymphocyte levels (Figure 7.1). However, the median percentage of $CD4^+CD25^+$ T-lymphocyte was significantly higher than the median percentage of $CD4^+CD25^-$ T-lymphocytes at day 1 post-treatment ($P<0.0001$) and at 5-7 weeks post-treatment ($P=0.0005$) (Figure 7.1). In comparison to baseline levels (246 cells/µl), an increasing trend in the $CD4^+$ count was observed at day 1

post-treatment (256 cells/µl), and at 5-7 weeks post-treatment (388 cells/µl), however this did not reach significance (P=0.32) (Table 7.1).

$CD8^+$ T-lymphocyte populations also showed a declining progression from baseline to 5-7 weeks post-treatment, with an increase in frequency only being noted in the $CD8^+CD25^+$ T-cell subset at day 1 post-treatment. The analysis of variance revealed a non-significant variation in the frequency of circulating total $CD8^+$, $CD8^+CD25^+$ and $CD8^+CD25^-$ T-lymphocyte during the follow-up period. The prevalence of circulating $CD25^+$ expressing $CD8^+$ T-lymphocytes was also significantly higher than $CD8^+CD25^-$ at day 1 post-treatment (P=0.0001) and at 5-7 weeks post-treatment (P=0.0049) (Figure 7.1). In comparison to the baseline total $CD8^+$ T-cell concentration (149 cells/µl) levels, patients at day 1 post-treatment showed a decline in the total $CD8^+$ T-cell concentration (119 cells/µl); however, this was followed by an increase in frequency at 5-7 weeks post-treatment (132 cells/µl), although not to the same level as that observed at baseline (Table 7.1). This variation was not statistically significant (P= 0.59) (Table 7.1).

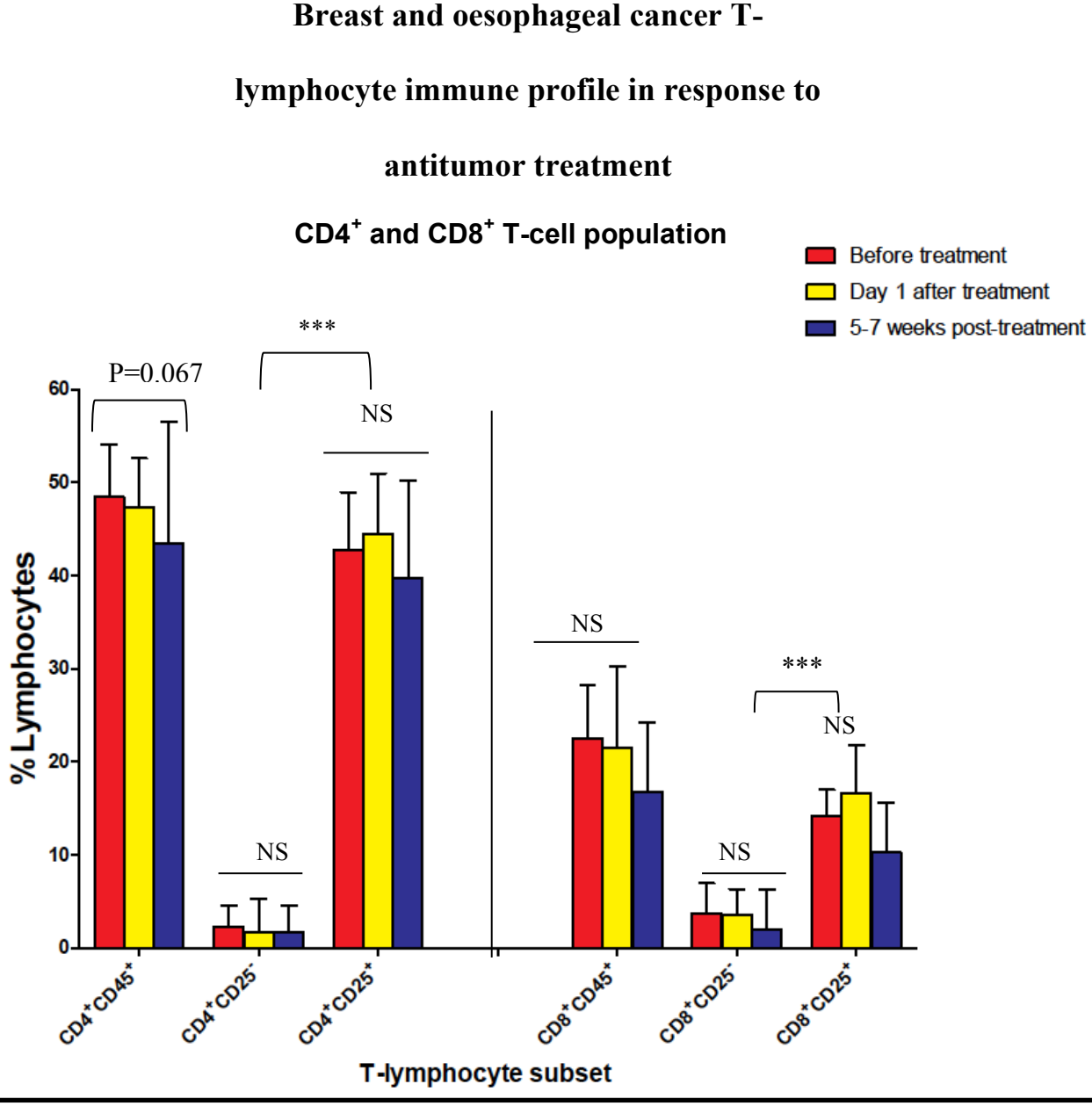


Figure 7.1: The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in breast and oesophageal cancer patients before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

Table 7.1: The $CD4^+$ and $CD8^+$ T-cell counts (cells/µl) in breast and oesophageal cancer patients before treatment, at day 1 post-treatment and 5-7 weeks post-treatment

Cell type	Median ±IQR			
	Time point 1	Time point 2	Time point 3	
Cells/µl	Before treatment	Day 1 post-treatment	5-7 weeks post-treatment	Statistical significance
Total $CD4^+$ count	246 (172-425	256 (154-447)	388 (212-567)	P=0.32
Total $CD8^+$ count	149 (57-306)	119 (82-197	132 (82-202)	P=0.59
CD4:CD8 ratio	2.0 (1.3-2.6)	2.2 (1.0-3.3)	3.14 (1.14-3.39	P=0.71
$CD4^+CD25^+CD127^+$	10 (4-21)	5 (1-11)§	2 (1-21)	P=0.037*
$CD4^+CD127^+FoxP3^+$	0 (0-0.1)	0 (0)	0(0)	P=0.420
$CD4^+CD25^+FoxP3^+$	0 (0.02-0.5)	0.1 (0.4-0.8)	0.1 (0-0.3)	P=0.715
$CD8^+CD25^+CD127^+$	34 (18-64)	24 (12-38)	15 (9-53)¶	P=0.045*
$CD8^+CD25^+FoxP3^+$	0 (0-1)	0 (0-4)	0(0)	P=0.83
$CD8^+CD127^+FoxP3^+$	0 (0-0.1)	0 (0)	0(0)	P=0.2597

Statistically significant difference between time point 1 and 2 =§ (P=0.05)
Statistically significant difference between time point 1 and 3=¶ (P=0.045)
Statistically significant difference in variation (P>0.05) between time point 1, 2 and 3*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

7.2.1.2 Detection of circulating CD4$^+$ and CD8$^+$ Treg cell subsets before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment

A downward trend was observed in the frequency of $CD4^+CD25^+FoxP3^+$, $CD4^+CD25^+CD127^+$, $CD8^+CD127^+Fox3^+$ and $CD8^+CD25^+CD127^+$ Treg cell percentage at day 1 post-treatment and 5-7 weeks post-treatment (Figure 7.2). However, these variations in frequency did not reach significance. Interestingly, a similar trend was observed in the circulating $CD4^+CD25^+CD127^+$ (P=0.037) and $CD8^+CD25^+CD127^+$ (P=0.045) Treg cell concentrations (cells/µl) (Table 7.1), with these subsets achieving significance.

An upward trend was observed in the median percentage of $CD8^+CD25^+FoxP3^+$ and $CD4^+CD127^+FoxP3^+$ Treg cells from baseline to day 1 post-treatment, however this was followed by a decline to baseline levels at 5-7 weeks post-treatment (Figure 7.2). These variations were not statistically significant.

Interestingly, the median percentage and cell concentration (cells/µl) of circulating $CD4^+CD25^+CD127^+$ Treg cells were significantly higher than $CD4^+CD25^+FoxP3^+$ Treg cells at day 1 post-treatment (P<0.0001) and at 5-7 weeks post-treatment (P=0.002) (Figure 7.2 and Table 7.1). Furthermore, the prevalence of $CD4^+CD25^+CD127^+$ Treg cell was also significantly higher than $CD4^+CD127^+FoxP3^+$ Treg cells at day 1 post-treatment (P<0.0001) and 5-7 weeks post-treatment (P=0.0020) (Figure 7.2 and Table 7.1).

The median percentage and cell concentration of $CD8^+CD25^+CD127^+$ Treg cells were also found to be significantly higher than $CD8^+CD25^+FoxP3^+$ Treg cell at baseline (P=0.0001), at day 1 post-treatment (P<0.0001) and at 5-7 weeks post-treatment (P=0.0039) (Figure 7.2 and Table 7.1). Moreover, the median percentage and cell concentration of $CD8^+CD25^+CD127^+$ Treg cell subset was also found to be significantly higher than $CD8^+CD127^+FoxP3^+$ at

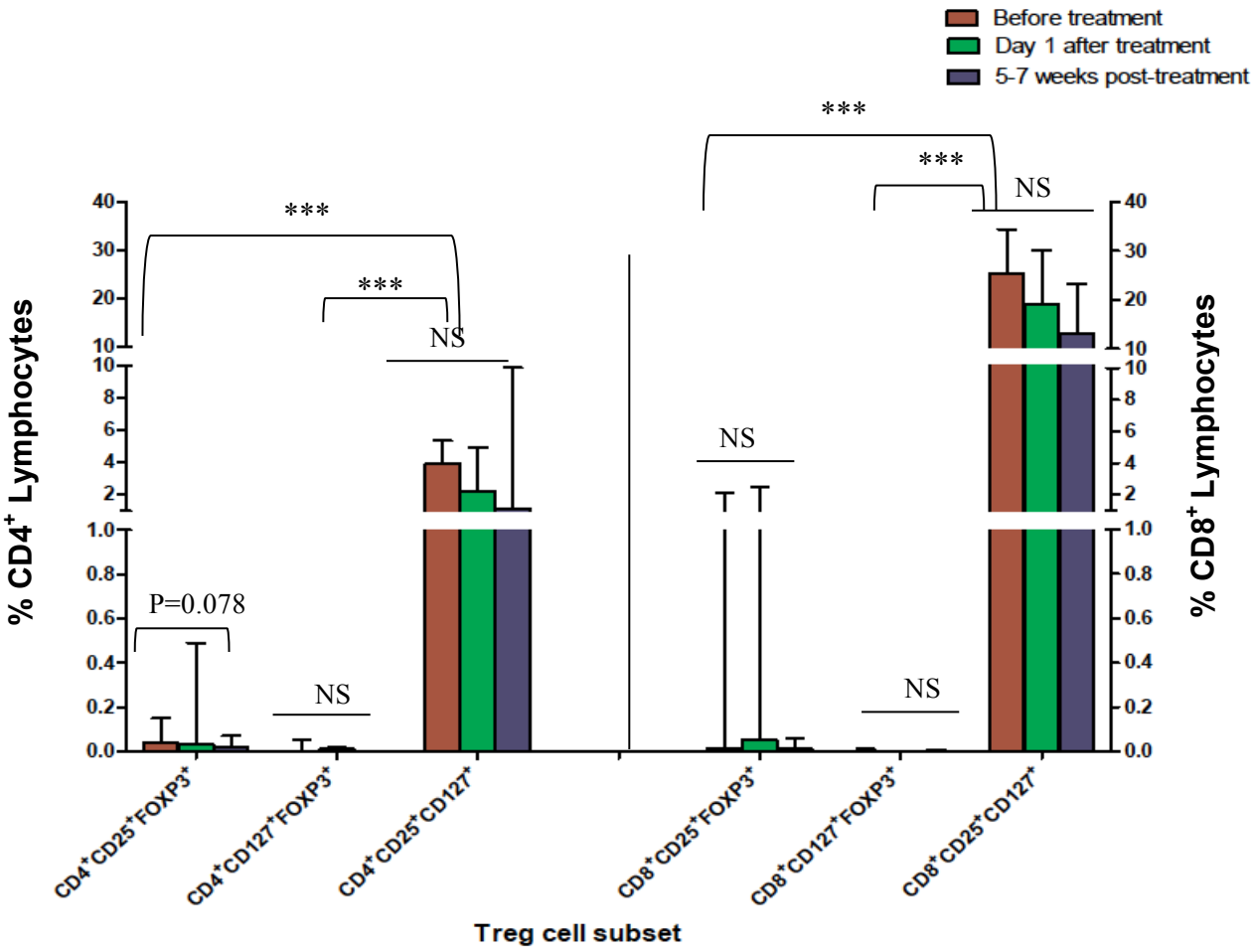


Figure 7.2: The frequency distribution of $CD4^+$ and $CD8^+$ Treg subsets in breast and oesophageal cancer patients before treatment (brown bar), at day 1 after treatment (green bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of Treg subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

baseline (P< 0.0001), at day 1 post-treatment (P<0.0001) and at 5-7 weeks post-treatment (P=0.0039) (Figure 7.2 and Table 7.1). A large interquartile range was observed in the $CD8^+CD25^+FoxP3^+$ Treg cell subset at baseline and day one after treatment (Figure 7.2), which was driven by the presence of high frequency of $CD8^+CD25^+FoxP3^+$ Treg cell in patients with advanced disease. By 5-7 week follow-up these participants had demised or were lost to follow-up (Figure 7.2). Due to the small sample size we were unable to further investigate the effect of the stage of disease on the Treg subset.

7.2.1.3 Effect of antitumor treatment on breast and oesophageal cancer $CD4^+$:$CD8^+$ T-lymphocyte, $CD4^+$ Treg: total $CD4^+$ and $CD8^+$ Treg: total $CD8^+$ ratio

In order to assess the overall effect of antitumor treatment on immunity, this study investigated the variation in the ratio between the total $CD4^+$ or $CD8^+$ Treg cells percentage and the total $CD4^+$ ($CD4^+CD45^+$) and total $CD8^+$ ($CD8^+CD45^+$) T-lymphocyte percentages. Furthermore the variation in the total $CD4^+$: total $CD8^+$ T-cell concentration ratio across the different time points under investigation was assessed.

The total $CD4^+$ Treg cell percentage at each time point was calculated by tallying the percentage $CD4^+CD25^+FoxP3^+$, $CD4^+CD25^+CD127^+$ and $CD4^+CD127^+FoxP3^+$ Treg cell subsets observed at the respective time points. While the total $CD8^+$ Treg cell percentage at each time point was calculated by tallying the percentage $CD8^+CD25^+FoxP3^+$, $CD8^+CD25^+CD127^+$ and $CD4^+CD127^+FoxP3^+$ Treg cell subsets.

A non-significant declining trend was observed in the $CD4^+$ Treg: total $CD4^+$ T-cell ratio at day 1 post-treatment and at 5-7 weeks post-treatment (P=0.499), in comparison to the baseline (Figure 7.3). A similar trend was observed in the $CD8^+$ Treg: total $CD8^+$ T-cell ratio, however it also did not reach significance (P=0.40) (Figure 7.3). Interestingly, The $CD8^+$

Treg: total $CD8^+$ T-cell ratio was higher than the $CD4^+$Treg: total $CD4^+$ T-cell ratio at all time points (Figure 7.3).

Unlike the downward trend observed in the $CD4^+$ Treg: total $CD4^+$ T-cell ratio and $CD8^+$ Treg: total $CD8^+$ T-cell ratio, the total $CD4^+$: total $CD8^+$ T-cell concentration ratio was seen to have an upward trend over the follow-up period, moving from a ratio of 2.0 (IQR: 1.3-2.6) at baseline and peaking at a ratio of 3.14 (IQR: 2.4-4.3) at 5-7 weeks post-treatment (Table 7.1). However, this trend was not statistically significant (P=0.71).

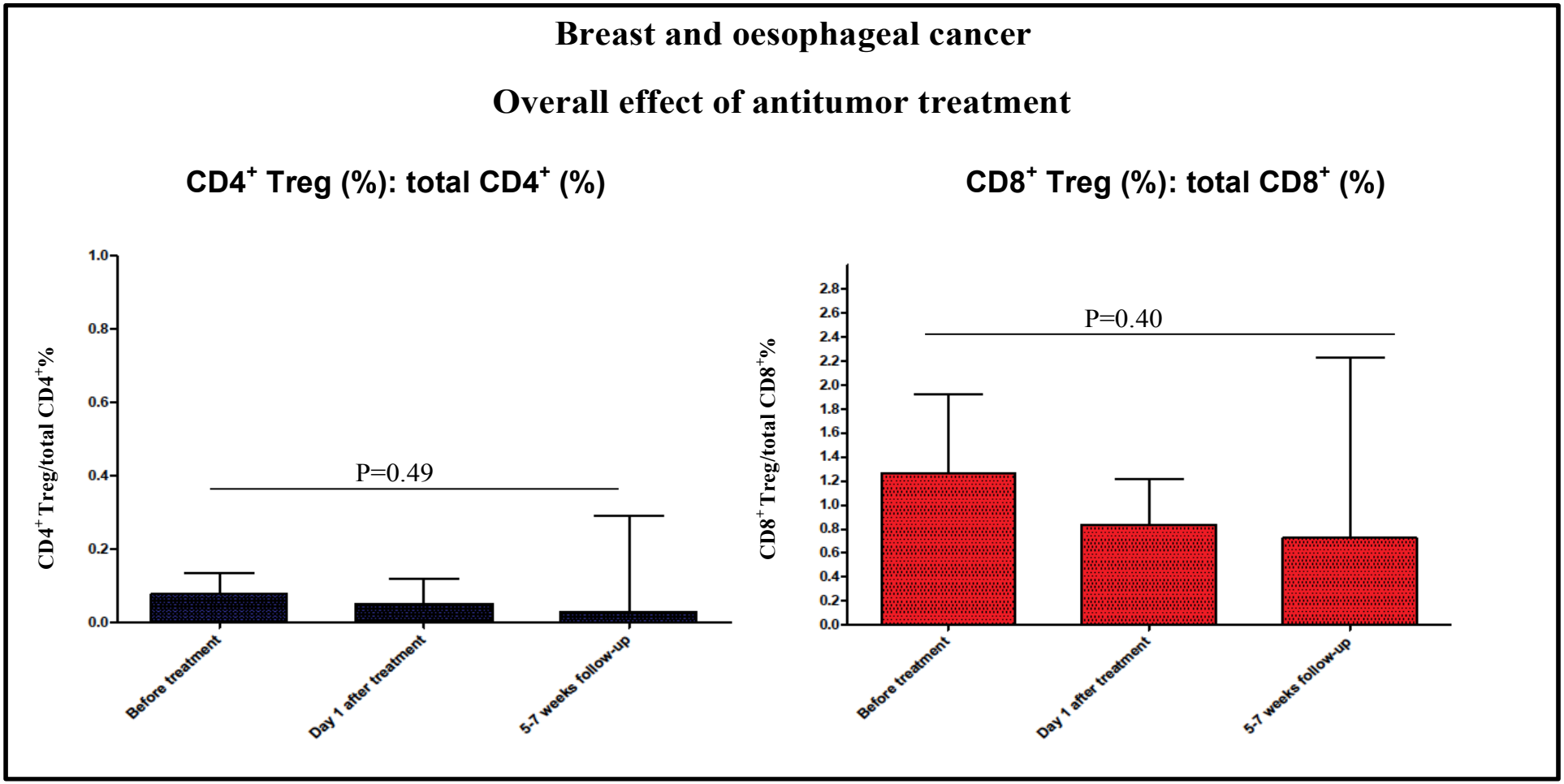


Figure 7.3: The frequency distribution of $CD4^+$ Treg (%): total $CD4^+$(%) and $CD8^+$ Treg (%): total $CD8^+$(%) ratio in breast and oesophageal cancer patients before treatment, at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median % ± IQR of $CD4^+$ Treg/total $CD4^+$ or $CD8^+$ Treg/total $CD8^+$ ratio detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001).

7.2.2 Breast cancer patients: Surgical versus chemotherapeutic intervention

In order to assess the effect of surgical and neo-adjuvant chemotherapeutic intervention on the circulating T-lymphocyte subsets in patients with breast cancer, the prevalence of T-lymphocytes was quantified before surgery or chemotherapy, at day 1 post-surgery or chemotherapy and at 5-7 weeks post-surgical or chemotherapeutic intervention.

Breast cancer patients who were eligible for neo-adjuvant chemotherapy presented with a more progressive stage of disease as compared to those eligible for surgical intervention. All chemotherapy patients received combination therapy as determined by the treating oncologist, which consisted of cyclophosphamide in combination with adriamycin 5FU or methotrexate. Of the patients who were eligible for surgical intervention, 94% received a modified radical mastectomy (MRM) with or without axillary clearance, while only 5.3% of patients received a lumpectomy. This was generally followed by a course of adjuvant chemotherapy, which was commenced no later than 7 weeks post surgery.

7.2.2.1 Detection of Non-Treg T-lymphocyte population in breast cancer patients receiving surgical or chemotherapeutic intervention, before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment

Patients who received surgical treatment showed a significant variation in the circulating levels of total $CD4^+$ (P=0.037) and $CD4^+CD25^+$ (P=0.009) T-lymphocyte percentages between the different time-points (Figure 7.4). The total $CD4^+$ T-lymphocyte percentage observed before treatment 48.49% (IQR: 39.29-56.16) remained stable at day 1 post surgery (48.91%; IQR 38.87-54.97), however it declined to 47.17% (IQR: 40-56.47%) at 5-7 weeks post-treatment (Figure 7.4). Similarly, the median $CD4^+CD25^+$ T-lymphocyte percentage

showed a downward trend from 45.85% (IQR: 37.42-51.63%) before treatment to 41.49% (IQR: 38.8-52.12) at 5-7 weeks post-treatment. A post-hoc pairwise comparison did not show a significant difference between the time points. Interestingly, the total $CD4^+$ T-cell concentration showed an increasing trend during this period from 260 cells/µl before treatment to 440 cells/µl at 5-7 weeks post-treatment (P=0.62) (Table 7.2).

When the total $CD4^+$ percentage in surgical patients was stratified according to the $CD25^+$ expression at different time points, a significantly higher median percentage of $CD4^+CD25^+$ T-lymphocytes were observed before treatment (P≤0.001), at day 1 after treatment (P≤0.001) and at 5-7 weeks post-treatment (P=0.0039), as compared to $CD4^+CD25^-$ T-lymphocytes (Figure 7.4).

A downward trend in the median percentage of total $CD8^+$ T-lymphocyte was observed at 5-7 weeks post surgery (16.81; IQR 14.4-22.82) as compared to baseline levels (22.43%; IQR 16.14-26.83). However, although a decline was also observed in the total $CD4^+$ T-cell population, the decline observed in the total $CD8^+$ T-cell subset was more acute and severe, as compared to that observed in the total $CD4^+$ T-cell subset. This acute decline is best observed at day 1 post-treatment, where the median total $CD8^+$ T-lymphocyte percentage declined from 22.43% to 19.66% (15.75%-25.67%), as apposed to the total $CD4^+$ T-cell population which remained stable at day 1 post-treatment (48.49% before treatment versus 48.91% at day 1 post-treatment) (Figure 7.4).

The decline observed in the median total $CD8^+$ T-lymphocyte percentage was also associated with a decline in the total $CD8^+$ T-cell concentration from 187 cells/µl at baseline to 160 cells/µl at day 1 post-treatment (Table 7.2). At 5-7 weeks post-treatment, the $CD8^+$ count

remained similar to levels observed at day 1 post-treatment (160 cells/µl). This variation was not statistically significant (P=0.48)

A significantly higher proportion of $CD8^+$ T-lymphocytes expressed $CD25^+$ receptor before treatment ($P \leq 0.002$), after treatment ($P \leq 0.001$) and at 5-7 weeks post-treatment (P=0.0117), as compared to $CD8^+CD25^-$ T-lymphocytes (Figure 7.4). Although a decline was observed in the frequency of circulating $CD8^+CD25^-$% and $CD8^+CD25^+$% at 5-7 weeks post-treatment, in comparison to baseline levels, this was not statistically significant (Figure 7.4).

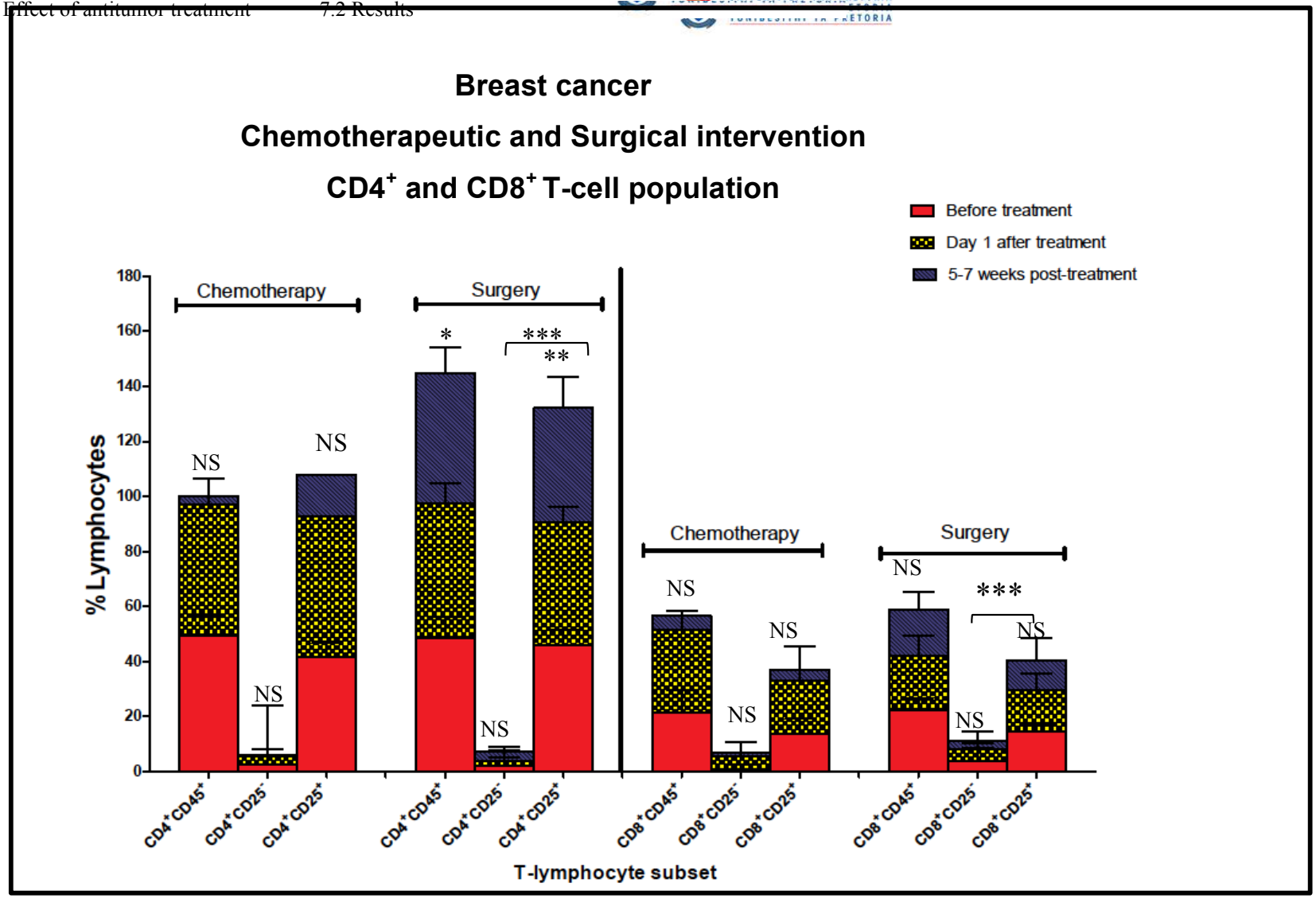


Figure 7.4: The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in breast cancer patients receiving chemotherapy and surgical intervention, before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001).

Patients receiving chemotherapy demonstrated a similar median total $CD4^+$ T-lymphocyte percentage at day 1 post-treatment as compared to baseline levels, while the remaining non-Treg T-lymphocyte subsets increased in frequency at day 1 post-treatment (Figure 7.4). This was followed by a substantial decline in the median percentage of all non-Treg T-cell subsets at 5-7 weeks post-treatment, far below levels observed at baseline (Figure 7.4). Due to the small sample size in the chemotherapy cohort, statistical analysis could not be conducted.

The circulating total $CD4^+$ T-lymphocyte concentration in patients receiving chemotherapy was shown to decline from 292 cells/µl (IQR: 222-345 cells/µl) at baseline, to 234 cells/µl (154-281 cells/µl) at day 1 post-treatment (Table 7.2). This was followed by a substantial decline in the $CD4^+$ count (39 cells/µl) at 5-7 weeks post-treatment (Table 7.2). Similarly, the $CD8^+$ T-lymphocyte concentration observed at baseline (151 cells/µl IQR: 135-153) declined to 109 cells/µl (IQR: 98-232) at day 1 post-treatment with a further decline observed at 5-7 weeks post-treatment (55 cells/µl) (Table 7.2).

Patients receiving chemotherapy displayed a higher median percentage of $CD25^+$ expressing $CD4^+$ T-lymphocytes before treatment, at day 1 post-chemotherapy and 5-7 weeks post-chemotherapy as compared to $CD4^+CD25^-$ T-lymphocyte (Figure 7.4). Similarly, A higher proportion of $CD8^+CD25^+$ T-lymphocytes (%) was observed before chemotherapy and at day 1 post-chemotherapy as compared to $CD8^+CD25^-$ T-lymphocyte (%). Due to the small sample size, statistical analysis could not be conducted (Figure 7.4).

In comparison to chemotherapy patients, surgical patients displayed a significantly higher median $CD8^+CD25^-$ (P=0.05) T-lymphocyte percentage at baseline, however the remaining

non-Treg T-lymphocyte (%) profiles were similar (Figure 7.4). Interestingly, at day 1 post-treatment chemotherapy patients displayed a higher median percentage of almost all the non-Treg cell subsets, with surgical patients only displaying a higher total $CD4^+$ T-lymphocyte percentage (Figure 7.4). At 5-7 weeks post-treatment the T-lymphocyte levels in surgical patients were substantially higher than that observed in chemotherapy patients; however, the small sample size in the chemotherapy group prevented further statistical analysis (Figure 7.4).

When the T-lymphocyte cell concentrations were compared, chemotherapy patients were found to have a higher $CD4^+$ T-lymphocyte concentration (292 cells/µl, IQR: 222-345) as compared to surgical patients (260 cells/µl, IQR: 150-559) (Table 7.2) at baseline. However, surgical patients displayed a substantially higher $CD4^+$ T-lymphocyte count at day 1 post-treatment (396 cells/µl versus 234 cells/µl) and at 5-7 weeks post-treatment (440 cells/µl versus 39 cells/µl), as compared to chemotherapy patients. Furthermore, in comparison to chemotherapy patients, surgical patients were found to have a substantially higher level of $CD8^+$ T-lymphocytes at baseline (187 cells/µl versus 151 cells/µl), at day 1 post-treatment (161 cells/µl versus 109 cells/µl) and at 5-7 weeks post-treatment (160 cells/µl versus 55 cells/µl).

Table 7.2: The $CD4^+$ and $CD8^+$ T-cell counts (cells/µl) in breast cancer patients receiving surgical or chemotherapeutic intervention; before treatment, at day 1 post-treatment and 5-7 weeks post-treatment

Surgical patients:				
Cell type	**Median ±IQR**			
	Time point 1	**Time point 2**	**Time point 3**	
Cells/µl	**Before treatment**	**Day 1 post-treatment**	**5-7 weeks post-treatment**	**Statistical significance (p)**
Total $CD4^+$ count	260 (150-559)	396 (213-473)	440 (252-597)	0.62
Total $CD8^+$ count	187 (61-254)	161 (88-203)	160 (114-203)	0.48
$CD4^+$:$CD8^+$ ratio	2.2 (1.4-3.5)	2 (1.5-3.4)	3.2 (1.7-3.5)	0.74
$CD4^+CD25^+CD127^+$	10 (4-22)	8 (2-14)	2 (1-14)	0.33
$CD4^+CD127^+FoxP3^+$	0 (0)	0 (0)	0 (0)	0.36
$CD4^+CD25^+FoxP3^+$	0 (0)	0 (0)	0 (0)	0.278
$CD8^+CD25^+CD127^+$	45 (18-72)	27 (16-51)	16 (11-37)	0.055*
$CD8^+CD25^+FOXP3^+$	0 (0)	0 (0)	0 (0)	0.54
$CD8^+CD127^+FoxP3^+$	0 (0)	0 (0)	0 (0)	0.30
Chemotherapy patients				
Total $CD4^+$ count	292 (222-345)	234 (154-281)	39 (39-39)	0.38
Total $CD8^+$ count	151 (135-153)	109 (98-232)	55 (55-55)	0.56
$CD4^+$:$CD8^+$ ratio	2.0 (1.4-2.1)	2 (1-3)	0.7 (0.7-0.7)	0.11
$CD4^+CD25^+CD127^+$	10 (2-20)	7 (4-18)	1 (1-1)	0.22
$CD4^+CD127^+FoxP3^+$	0 (0)	0 (0)	0 (0)	0.50
$CD4^+CD25^+FoxP3^+$	0.5 (0.2-1)	1 (0-5)	0 (0)	0.60
$CD8^+CD25^+CD127^+$	47 (38-63)	21 (12-38)	2 (2-2)	0.59
$CD8^+CD25^+FOXP3^+$	5 (0-9)	6 (0-6)	0 (0)	0.96
$CD8^+CD127^+FoxP3^+$	0 (0)	0 (0)	0 (0)	1

Statistically significant difference between time point 1 and 2 =§ (P=0.05)
Statistically significant difference between time point 1 and 3=¶ (P=0.045)
Statistically significant difference in variation (P>0.05) between time point 1, 2 and 3*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

7.2.2.2 Detection of Treg CD4$^+$ and CD8$^+$ Treg cell subsets in breast cancer patients receiving chemotherapy and surgical treatment intervention; before treatment, at day 1 after treatment and at 5-7 weeks post-treatment intervention

A variety of CD4$^+$ and CD8$^+$ Treg cell populations were observed in both treatment groups at baseline (Figure 7.5). Surgical patients displayed a significantly higher median percentage and circulating concentration (cells/µl) of CD4$^+$CD25$^+$CD127$^+$ Treg cells as compared to both CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (P<0.0001) and CD4$^+$CD127$^+$FoxP3$^+$ (P< 0.0001) Treg cell subsets before surgery (Figure 7.5 and Table 7.2). The median CD4$^+$CD25$^+$CD127$^+$ Treg cell percentage and circulating concentration (cells/µl) remained significantly elevated in comparison to CD4$^+$CD25$^+$FoxP3$^+$ at day 1 post-treatment (P=0.0005) and at 5-7 weeks post-treatment (P=0.0059) (Figure 7.5 and Table 7.2). Similarly, the median CD4$^+$CD25$^+$CD127$^+$ Treg cell percentage and circulating concentration (cells/µl) was also significantly elevated in comparison to CD4$^+$CD127$^+$FoxP3$^+$ at day 1 post-treatment (P=0.001) and 5-7 weeks post-treatment (P=0.0107) (Figure 7.5 and Table 7.2). The median percentage of CD4$^+$CD25$^+$FoxP3$^+$ Treg was significantly higher than CD4$^+$CD127$^+$FoxP3$^+$ at day 1 post-treatment (P=0.0010) and at 5-7 week post-treatment (P=0.0107) (Figure 7.5).

The CD8$^+$CD25$^+$CD127$^+$ Treg cell prevalence and circulating concentration in surgical patients was also found to be significantly higher than CD8$^+$CD25$^+$FoxP3$^+$ Treg cells (P<0.0001) and CD8$^+$CD127$^+$FoxP3$^+$ (P<0.0001) Treg cells before surgery, at day 1 post-surgery [CD8$^+$CD25$^+$FoxP3$^+$ Treg cells (P=0.0001), CD8$^+$CD127$^+$FoxP3$^+$ Treg cells (P=0.0001)] and at 5-7 weeks post-surgery [CD8$^+$CD25$^+$FoxP3$^+$ Treg cells (P=0.0051), CD8$^+$CD127$^+$FoxP3$^+$ Treg cells (P=0.0051)] (Figure 7.5 and Table 7.2). The median prevalence of CD8$^+$CD25$^+$FoxP3$^+$ Treg cells was significantly higher than

$CD8^+CD127^+FoxP3^+$ Treg cells at baseline (P=0.025), at day 1 post-treatment (P=0.05) and 5-7 weeks post-treatment (P=0.02) (Figure 7.5).

Breast cancer patients who were eligible for chemotherapy displayed a higher median percentage of $CD4^+CD25^+CD127^+$ Treg cells (3.49, IQR: 0.8-7.37) as compared to $CD4^+CD127^+FoxP3^+$ (0.06, IQR: 0-0.11) and $CD4^+CD25^+FoxP3^+$(0.205, IQR: 0.13-0.31), at baseline, at day 1 post-treatment [($CD4^+CD25^+CD127^+$ Treg cells: 2.68 IQR 2.18-5.92), ($CD4^+CD127^+FoxP3^+$; 0.01 IQR 0-0.02), ($CD4^+CD25^+$FoxP3; 0.49 IQR 0.05-1.76)] and at 5-7 weeks post-treatment [($CD4^+CD25^+CD127^+$ Treg cells: 0.5), ($CD4^+CD127^+FoxP3^+$: 0), ($CD4^+CD25^+$FoxP3: 0.01)] (Figure 7.5).

Similarly, the median percentage of $CD8^+CD25^+CD127^+$ Treg cells was higher than $CD8^+CD25^+FoxP3^+$ Treg cells and $CD8^+CD127^+FoxP3^+$ Treg cells and $CD8^+CD25^+FoxP3^+$ Treg cells at baseline, at day 1 post-treatment and 5-7 weeks post-treatment (Figure 7.5). Statistical analysis could not be performed due to the small sample size.

Surgical patients demonstrated a statistically significant decline in the median prevalence of $CD8^+CD25^+CD127^+$ Treg cell from baseline levels to 5-7 weeks post-treatment (P=0.012), while chemotherapy patients demonstrated a downward trend in the circulating prevalence of $CD4^+CD25^+CD127^+$ Treg cell at 5-7 weeks post-treatment in comparison to baseline levels; however, this did not reach significance (P=0.097) (Figure 7.5). Although the remaining Treg cell subsets showed a declining trend from baseline to 5-7 weeks post-treatment, this also did not reach significance (Figure 7.5).

Chemotherapy patients were shown to have a higher prevalence of circulating $CD8^+CD25^+FoxP3^+$ (0.054) and $CD4^+CD25^+FoxP3^+$ (P=0.0157) Treg cells at baseline, in comparison to surgical patients (Figure 7.5). Although, chemotherapy patients appeared to display higher median $CD4^+$ and $CD8^+$ Treg levels at day 1 post-treatment, and lower $CD4^+$ and $CD8^+$ Treg levels at 5-7 weeks post-treatment, this could not be confirmed statistically due to the small sample size.

Interestingly, despite differences observed in the median percentages of $CD4^+$ and $CD8^+$ Treg cell population in surgical and chemotherapy patients, these two groups were found to have similar circulating concentrations of Treg cell counts at the different time points, irrespective of the treatment prescribed.

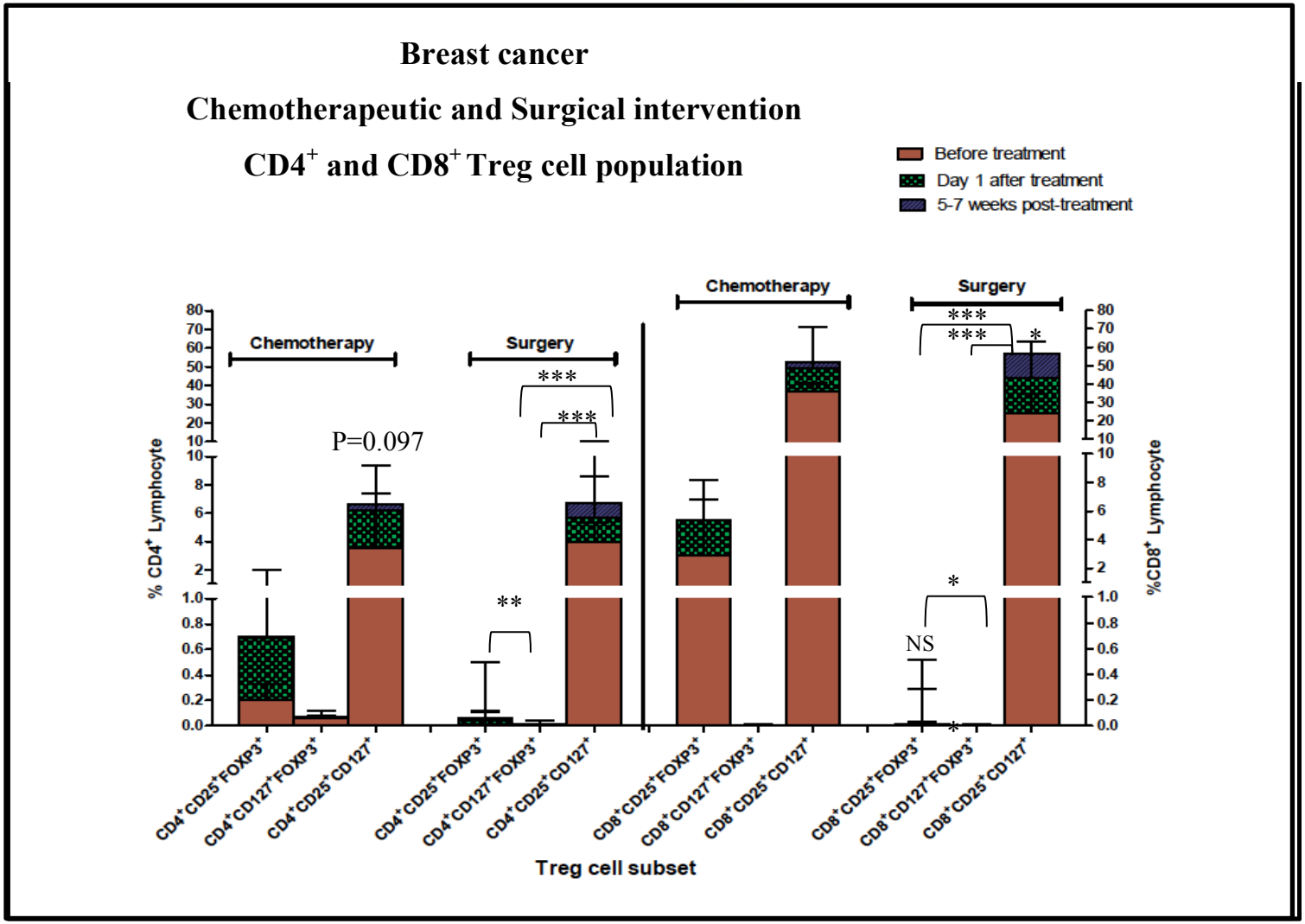


Figure 7.5: The frequency distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in breast cancer patients receiving chemotherapy and surgical intervention, before treatment (brown bar), at day 1 after treatment (green bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. $CD4^+CD25^+CD127^+$ and $CD8^+CD25^+CD127^+$ Treg cells were the most abundant Treg populations at all time intervals, in both surgical and chemotherapy patients. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

7.2.2.3 Effect of surgical and chemotherapeutic treatment on the CD4$^+$Treg: total CD4$^+$ T-cell ratio and the CD8$^+$Treg: total CD8$^+$ T-cell ratio in patients presenting with breast cancer

- **CD4$^+$Treg: total CD4$^+$ T-cell ratio**

Patients who received chemotherapeutic treatment showed an upward trend in the CD4$^+$Treg: total CD4$^+$T-cell ratio with a higher ratio found at the 5-7 weeks follow-up as compared to baseline levels (Figure 7.6). Interestingly, surgical patients demonstrated the opposite effect with a decreasing trend in the CD4$^+$(%): total CD4$^+$ T-cell ratio over time, and a lower CD4$^+$(%): total CD4$^+$ T-cell ratio being found at 5-7 weeks follow-up as compared to that observed at baseline (Figure 7.6). These findings however did not reach statistical significance. Although, in comparison to surgical patients, patients receiving chemotherapy displayed higher CD4$^+$(%):total CD4$^+$ T-cell ratios, this did not reach significance (before treatment: P=0.89, day 1 post-treatment =0.31; 5-7 week post-treatment).

- **CD8$^+$Treg: total CD8$^+$ T-cell ratio**

Chemotherapy patients showed a non-significant variation (P=0.47) in the CD8$^+$ Treg: total CD8$^+$ T-cell ratio during the follow-up period, with a decline in CD8$^+$ Treg: total CD8$^+$ T-cell ratio observed at day 1 post-treatment, which was followed by an increase in CD8$^+$ Treg: total CD8$^+$ T-cell ratio at 5-7 weeks post-treatment (Figure 7.7).

Surgical patients also displayed a non-significant variation in the $CD8^+$ Treg: total $CD8^+$ T-cell ratio during the follow-up period. A decline was observed in the $CD8^+$ Treg: total $CD8^+$ T-cell ratio at day 1 post-treatment (P=0.27) and at 5-7 weeks post-treatment (P=0.14).

In comparison to surgical patients, chemotherapy patients were found to have an increased $CD8^+$ Treg: total $CD8^+$ T-cell ratio before treatment (P=0.94) and at 5-7 weeks post-treatment (P=0.75) (Figure 7.7). However, surgical patients were found to have a higher $CD8^+$ Treg: total $CD8^+$ T-cell ratio at day 1 post-treatment (P=0.57), as compared to patients receiving chemotherapy (Figure 7.7). This was not statistically significant.

- **$CD4^+$: $CD8^+$ T-cell ratio**

Surgical patients were found to have an upward trend in the $CD4^+$: $CD8^+$ T-cell ratio over the follow-up period with patients having a higher $CD4^+$: $CD8^+$ T-cell ratio at day 1 post-treatment (2; IQR: 1.5-3.4) and at 5-7 weeks post-treatment (3.2, IQR: 1.7-3.5), in comparison to baseline levels (2.2, IQR: 1.4-3.5). However, this trend was not significant (Table 7.2)

However, chemotherapy patients showed a decreasing trend on the $CD4^+$: $CD8^+$ T-cell ratio over the follow-up period with a lower, $CD4^+$: $CD8^+$ T-cell ratio at 5-7 weeks post-treatment (0.7) as compared to day 1 post-treatment (2, IQR: 1-3) and 5-7 weeks post-treatment (2, IQR: 1.4-2.1) (Table 7.2).

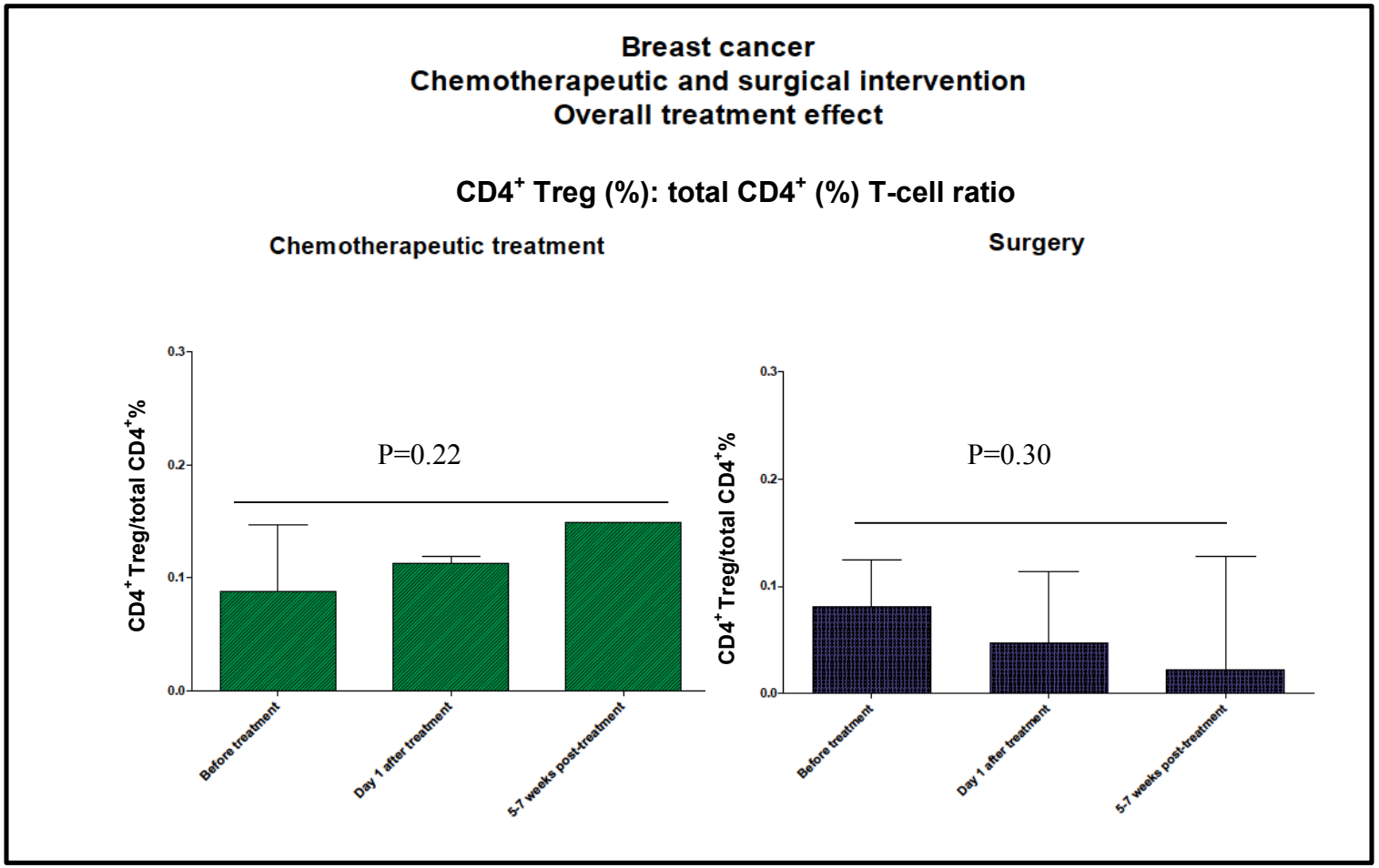


Figure: 7.6: The frequency distribution of $CD4^+$ Treg (%): total $CD4^+$(%) T-cell ratio in breast cancer patients receiving chemotherapy (green bars) and surgical (blue bars) intervention, before treatment , at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

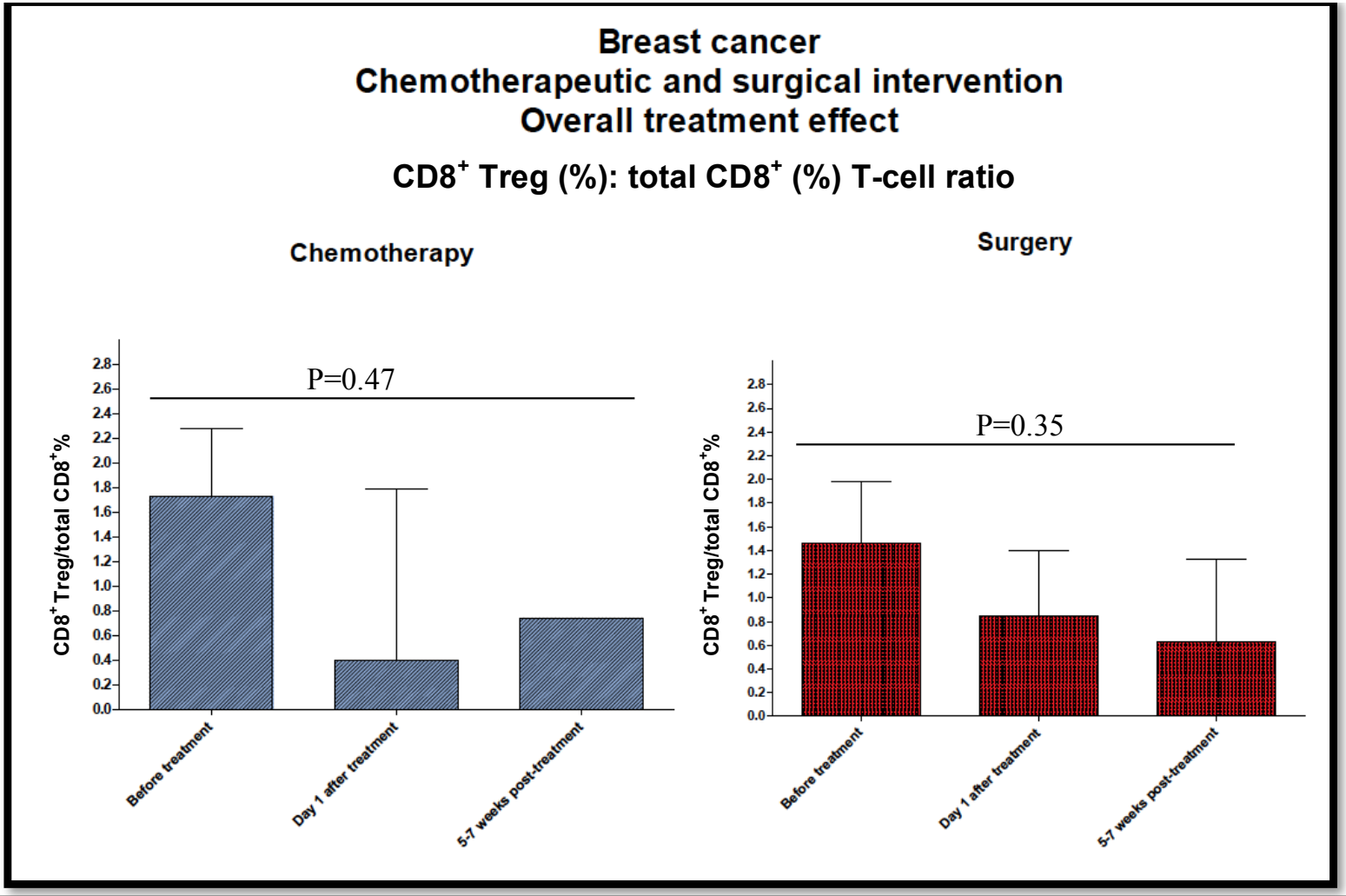


Figure: 7.7: The frequency distribution of CD8$^+$ Treg (%): total CD8$^+$(%) ratio in breast cancer patients receiving chemotherapy (blue bars) and surgical (red bars) intervention, before treatment , at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

7.2.3 Oesophageal cancer patients

In order to evaluate the effect of antitumor treatment on the T-lymphocyte profile in patients presenting with oesophageal cancer, the frequency of circulating T-lymphocyte subsets before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment were quantified. Due to the small sample size and high number of mortalities and loss to follow-up in this group, all results were analysed together irrespective of the type of treatment received.

7.2.3.1 Detection of $CD4^+$ and $CD8^+$ non-Treg T-lymphocyte population in oesophageal cancer patient before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment

In comparison to baseline levels, a decline was observed in the median percentage of the majority of $CD4^+$ and $CD8^+$ non-Treg cell populations (Figure 7.8), with $CD8^+CD25^+$ T-lymphocyte being the only T-cell population to show an increase at day 1 post-treatment (Figure 7.8). A significant variation in the median percentage of circulating $CD4^+CD25^-$ ($P=0.0072$) and $CD8^+CD25^-$ ($P=0.031$) T-lymphocytes were observed between baseline, day 1 post-treatment and 5-7 weeks post-treatment. Pairwise comparison revealed a significant decline in the median $CD4^+CD25^-$ ($P=0.011$), and $CD8^+CD25^-$ ($P=0.04$) at day 1 post-treatment, in comparison to levels observed before treatment (Figure 7.8). Substantial variation was also observed in the median percentage of $CD8^+CD25^+$ ($P=0.062$), but this did not reach significance. Only 1 patient was present for follow-up at 5-7 weeks post-treatment.

The $CD4^+CD25^+$ T-lymphocytes were significantly higher than the median $CD4^+CD25^-$ T-lymphocyte percentage before treatment (P=0.005); however, due to the small sample size at day 1 post-treatment (0.067) and 5-7 weeks post-treatment (P=0.31), a statistically significant difference was not observed (Figure 7.8).

Similarly, in comparison to the median percentage of $CD8^+CD25^-$ T-lymphocytes, $CD8^+CD25^+$ T^-lymphocytes were significantly higher before treatment (P=0.046); however, significance was lost at day 1 post-treatment (P=0.067) and 5-7 weeks post-treatment (P=0.31) (Figure 7.8).

The decline in the $CD4^+$ T-lymphocytes were also associated with a decline in the total $CD4^+$ T-lymphocyte concentration from 241 cells/µl (IQR: 210-416) to 112 cells/µl (IQR: 54-153) at day 1 post-treatment and 106 cells/µl at 5-7 weeks post-treatment (Table 7.3). Furthermore, the $CD8^+$ T-lymphocyte concentration also declined from 125 cells/µl to 65 cells/µl at day one post-treatment, however a mild recovery was observed at 5-7 weeks post-treatment (86 cells/µl) (Table 7.3).

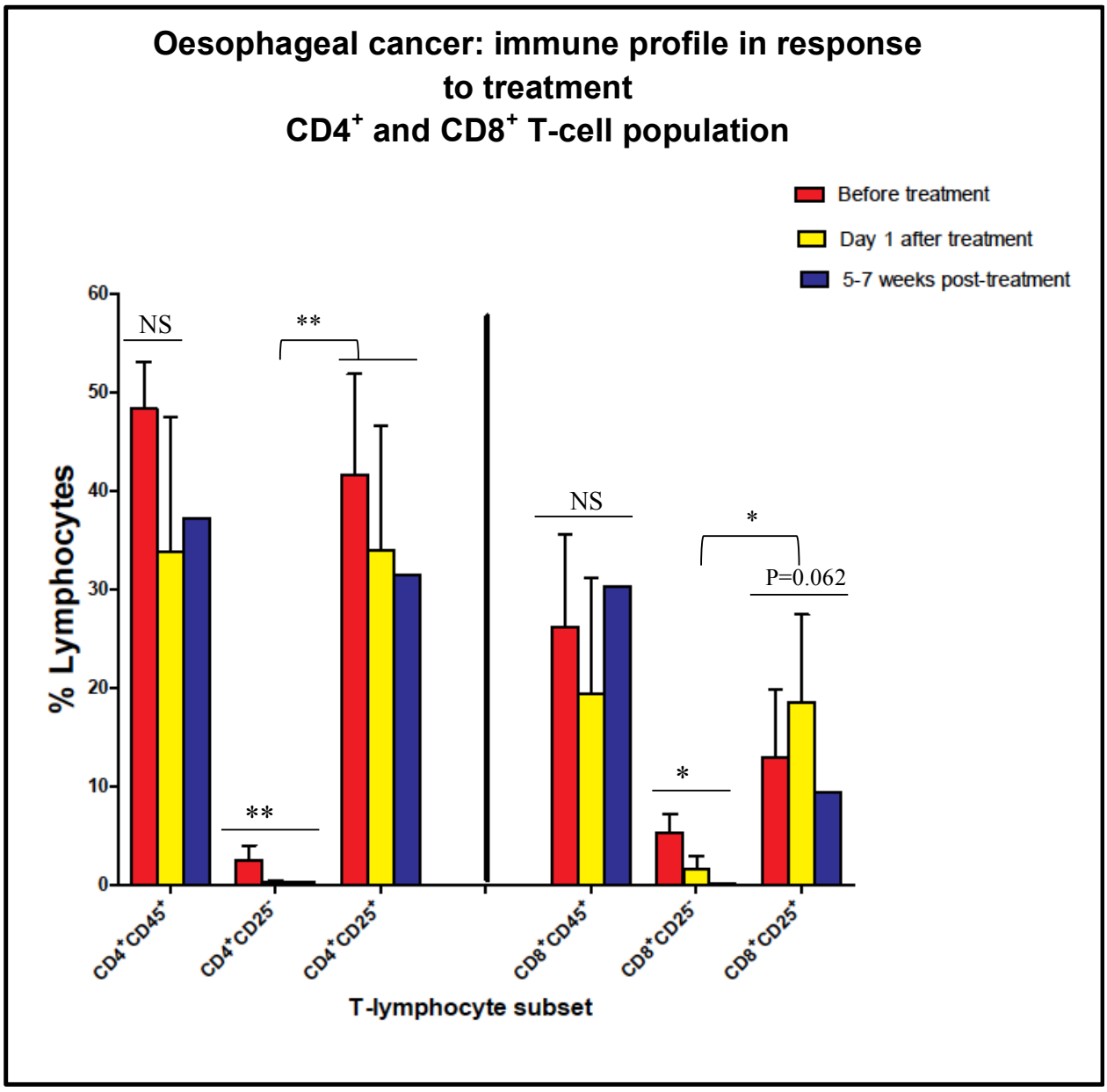


Figure 7.8: The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in oesophageal cancer patients before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

Table 7.3: The $CD4^+$ and $CD8^+$ T-cell counts (cells/µl) in oesophageal cancer patients before treatment, at day 1 post-treatment and 5-7 weeks post-treatment

Cell type	Median ±IQR			
	Time point 1	Time point 2	Time point 3	
Cells/µl	Before treatment	Day 1 post-treatment	5-7 weeks post-treatment	Statistical significance (P)
Total $CD4^+$ count	241 (210-416)	112 (54-153)	106 (106)	0.26
Total $CD8^+$ count	125 (74-180)	65 (38-96)	86 (86)	0.23
CD4:CD8 ratio	1.8 (1.2-2.5)	1.2 (0.5-2.4)	1.2 (1.2)	0.62
$CD4^+CD25^+CD127^+$	11 (7-22)	2 (0-4)	29 (29)	0.301
$CD4^+CD127^+FoxP3^+$	0 (0)	0 (0)	0 (0)	0.705
$CD4^+CD25^+FoxP3^+$	0 (0)	0 (0)	0 (0)	0.29
$CD8^+CD25^+CD127^+$	26 (18-35)	12 (6-22)	72 (72)	0.071
$CD8^+CD25^+FoxP3^+$	0 (0)	0 (0-5)	9 (9)	0.48
$CD8^+CD127^+FoxP3^+$	0 (0)	0 (0)	0 (0)	0.26

Statistically significant difference between time point 1 and 2 =§ (P=0.05)
Statistically significant difference between time point 1 and 3=¶ (P=0.045)
Statistically significant difference in variation (P>0.05) between time point 1, 2 and 3*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

7.2.3.2 *Detection of $CD4^+$ and $CD8^+$ Treg T-lymphocyte population in oesophageal cancer patients before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment*

Oesophageal cancer patients presented with a variety of $CD4^+$ and $CD8^+$ Treg cell subsets at the respective time points (Figure 7.9). The majority of $CD4^+$ and $CD8^+$ Treg cells showed an increasing trend from baseline to 5-7 week follow-up, with a significant variation in the trend observed in the $CD8^+CD25^+CD127^+$(P=0.0026), $CD8^+CD127^+FoxP3^+$(P=0.0139) and $CD4^+CD25^+CD127^+$ (P=0.025) Treg cell populations (Figure 7.9). Pairwise comparison revealed a significant increase in the median percentage of $CD8^+CD127^+FoxP3^+$ Treg at 5-7 weeks after treatment, in comparison to baseline levels (P=0.0443) and day 1 post-treatment (P=0.0455) (Figure 7.9).

A decrease in the $CD4^+CD25^+CD127^+$ Treg cell concentration (cells/µl) and $CD8^+CD25^+CD127^+$ was also observed at day 1 post-treatment (Table 7.3), however this was followed by a substantial increase in both cell subsets at 5-7 weeks post-treatment (Table 7.3). Furthermore, although $CD8^+CD25^+FoxP3^+$ Treg cells were undetectable before treatment and at day 1 post-treatment, an increase was observed at 5-7 weeks post-treatment (9 cells/µl) (Table 7.3).

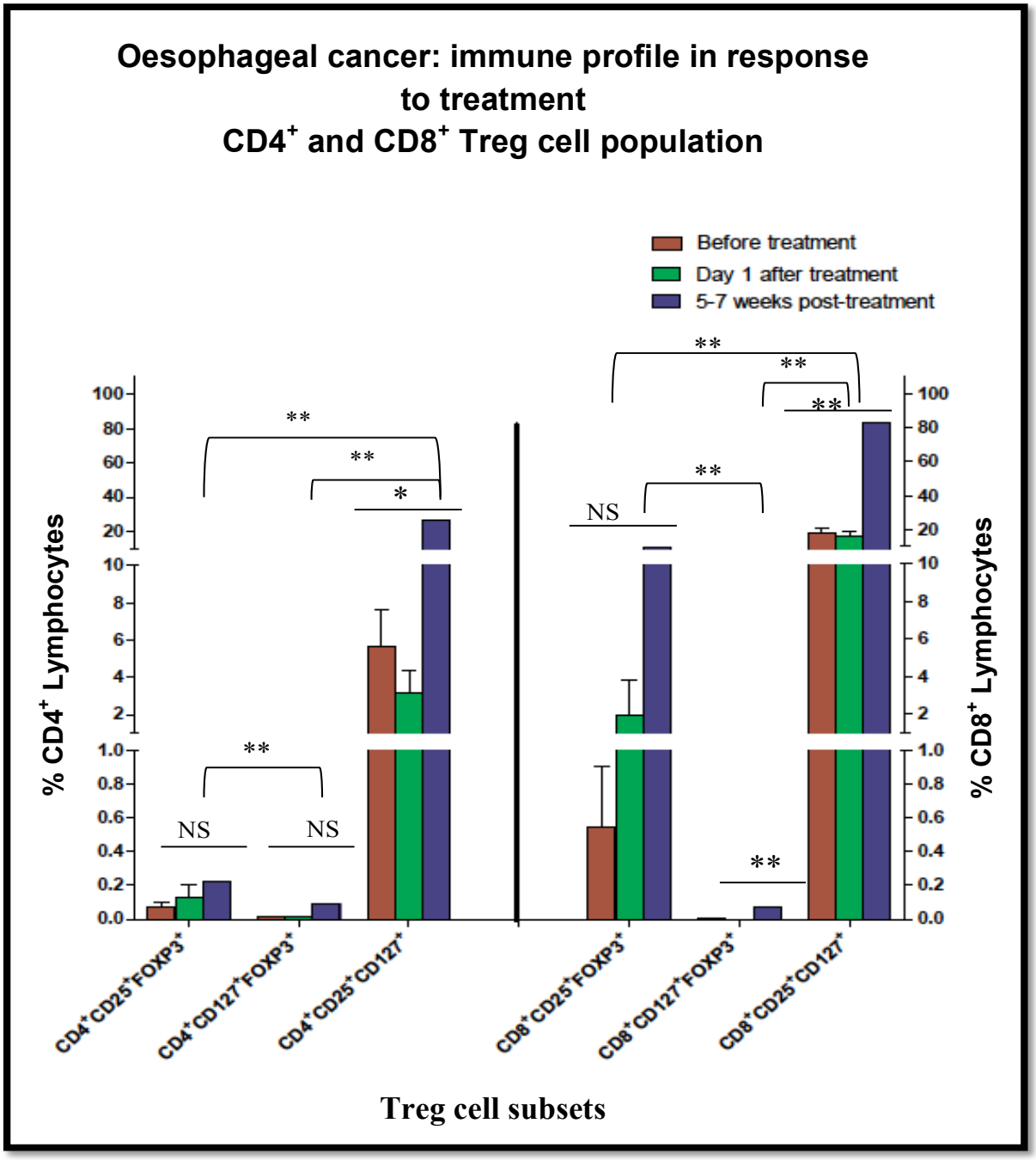


Figure 7.9: The frequency distribution of $CD4^+$ and $CD8^+$ Treg subsets in oesophageal cancer patients before treatment (brown bar), at day 1 after treatment (green bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of Treg subsets detected at different time points.

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

The median percentage and cell concentration (cells/µl) of $CD8^+CD25^+CD127^+$ Treg cell subset was significantly elevated as compared to the median percentage of $CD8^+CD25^+FoxP3^+$ Treg cells before treatment (0.0051), however significance was lost at day 1 post-treatment (P=0.067), and at 5-7 weeks post-treatment (P=0.31) (Figure 7.9 and Table7.3). Similarly, the median $CD8^+CD25^+CD127^+$ Treg percentage and circulating concentration (cells/µl) was significantly higher than $CD8^+CD127^+FoxP3^+$ Treg percentage before treatment, but non-significantly higher at day 1 post-treatment (P=0.069) and at 5-7 weeks post-treatment (P=0.31) (Figure 7.9 and Table 7.3). The median percentage of $CD8^+CD25^+FoxP3^+$ Treg cell was significantly higher than $CD8^+CD127^+FoxP3^+$ Treg cell (0.027) before treatment, with a non-significant difference observed at day 1 post-treatment (P=0.0679) and at 5-7 weeks post-treatment (P=0.317).

The median percentage and cell concentration of $CD4^+CD25^+CD127^+$ Treg cells was significantly higher than the median percentage of $CD4^+CD25^+FoxP3^+$ Treg cells (P=0.0069) and $CD4^+CD127^+FoxP3^+$ Treg cells (P=0.0059) before treatment. Furthermore, the median percentage of $CD4^+CD25^+FoxP3^+$ Treg cells was significantly higher than the median percentage of $CD4^+CD127^+FoxP3^+$ Treg cells (P=0.0076) before treatment, however statistical significance did not persist at day 1 post-treatment (P=0.0947) or at 5-7 weeks post-treatment (P=0.317).

7.2.3.3 Effect of antitumor treatment on the CD4$^+$Treg: total CD4$^+$ T-cell ratio and the CD8$^+$ Treg: total CD8$^+$T-cell ratio in patients presenting with oesophageal cancer

- **CD4$^+$ Treg: total CD4$^+$ T-cell ratio**

Oesophageal cancer patients displayed a statistically significant variation in the CD4$^+$Treg: total CD4$^+$ T-cell ratio between the time points under investigation (P=0.049). Due to the small sample size, pairwise analysis was not significant. The CD4$^+$Treg:total CD4$^+$ T-cell ratio at 5-7 weeks post-treatment was substantially higher than at baseline and at day 1 after treatment.

- **CD8$^+$Treg: total CD8$^+$ T-cell ratio**

The analysis of variance between the different time points suggested that the variation in the CD8$^+$Treg:total CD8$^+$ T-cell ratio was significant (P=0.0003). Pairwise analysis was not significant, however the CD8$^+$Treg:total CD8$^+$ T-cell ratio was substantially higher at 5-7 weeks post-treatment as compared to day 1 post-treatment and before treatment.

- **CD4$^+$: CD8$^+$ T-cell ratio**

Oesophageal cancer patients demonstrated a declining CD4$^+$: CD8$^+$ T-cell ratio from baseline levels (1.8; IQR, 1.2-2.5) to day 1 post treatment (1.2; IQR 0.5-2.4) (Table 7.3). This ratio was maintained at 5-7 weeks post-treatment (Table 7.3).

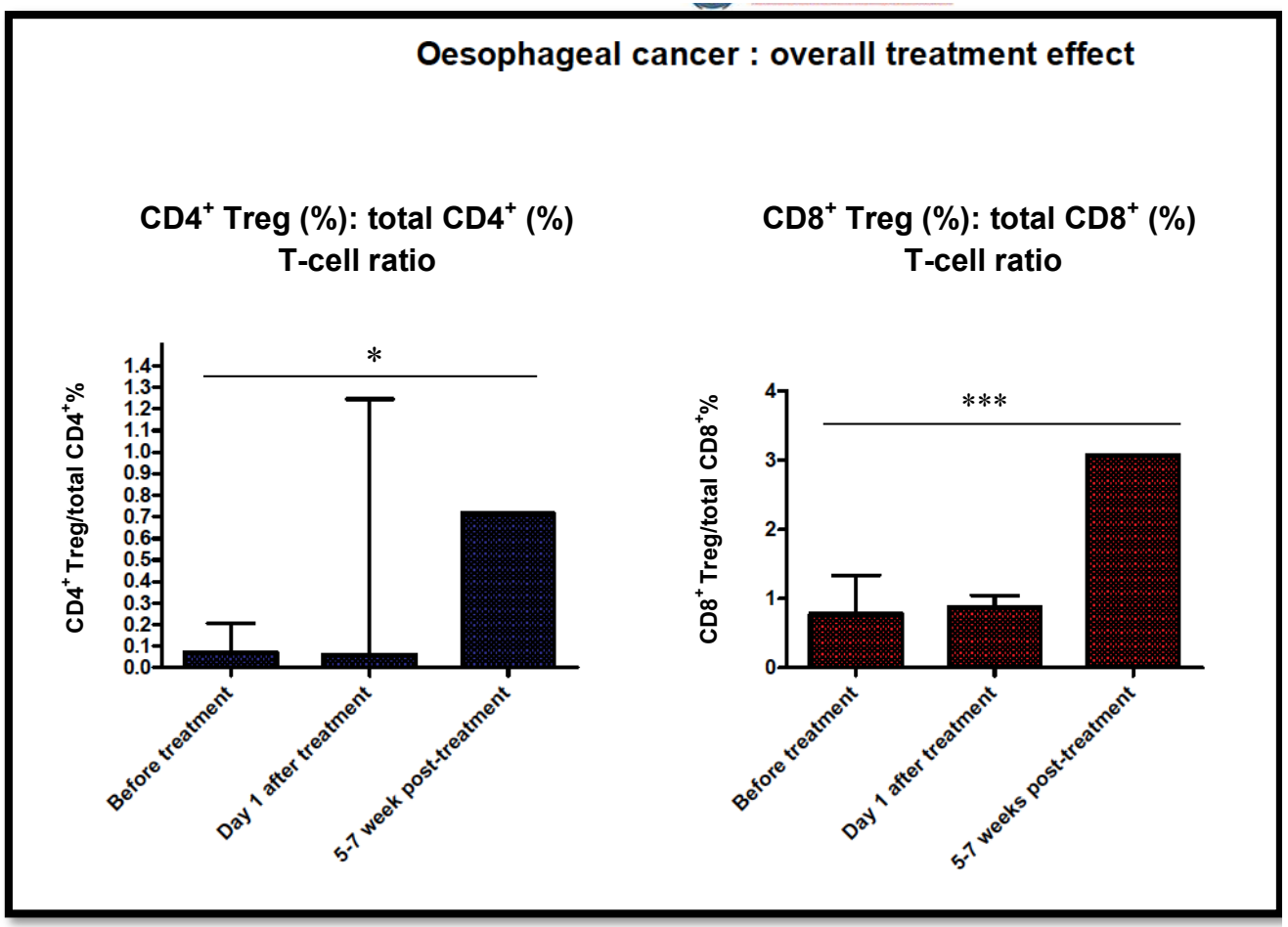


Figure: 7.10: The frequency distribution of $CD4^+$ Treg (%): total $CD4^+$(%) ratio and $CD8^+$ Treg (%): total $CD8^+$(%) ratio in oesophageal cancer patients receiving intervention, before treatment, at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. A significant variation was observed in the frequency of $CD4^+$Treg (%): total $CD4^+$(%) ratio (P=0.049), and $CD8^+$Treg (%): total $CD8^+$(%) ratio (P=0.0003) during the follow-up period. Pairwise comparison did not yield a significant result. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

7.3 Discussion

$CD4^+$ and $CD8^+$ T-lymphocyte populations have been shown to play a major role in the instigation, maintenance and regulation of antitumor immunity, with alterations in the distribution and ratios of these T-cell subsets serving as an indicator of immune impairment and disease progression. Surgery, chemotherapy and radiotherapy are the most common antitumor treatment modalities currently in use in this setting, and this study aimed to investigate the effect which antitumor treatment has on the T-lymphocyte immune profile. Furthermore, this study set out to determine whether antitumor treatment induces a shift in the T-cell immune profile towards one which promotes tumor progression and attenuates antitumor immunity (increased Treg cells: decreased non-Treg cells), or one which augments antitumor immunity and facilitates tumor eradication (decreased Treg cells: increased non-Treg cells).

Previous studies have demonstrated the effect of antitumor treatments on circulating T-lymphocyte subsets (Campbell et al. 1973, Colachio et al. 1994, De-en et al. 1998, Goldfarb et al. 2006, Gottschalk et al. 2012, Chu-yuan et al. 2013), however, to the best of my knowledge, this is the first report which assesses the influence of antitumor treatment on the circulating T-lymphocyte and T regulatory cell profile in South African patients presenting with breast and oesophageal cancer. Furthermore, this analysis was conducted on whole blood, as compared to a large proportion of previous studies where Ficoll-derived peripheral blood mononuclear cells were used (Verma et al 2013.) This ensures a better reflection of the circulating Treg and non-Treg immune status, as minimal manipulation of the participants' blood did not occur prior to analysis (Sellito et al. 2011).

When assessed together, anticancer treatment (surgery, chemotherapy and radiotherapy) was found to induce a non-significant decline in the $CD4^+$ and $CD8^+$ non-Treg and Treg cell profile (%) in breast and oesophageal cancer patients. This was associated with an increasing trend in the total $CD4^+$ T-cell concentration and a decreasing trend in the total $CD8^+$ T-cell concentration over the follow-up period. Consequently, breast and oesophageal cancer patients demonstrated an upward trend in the $CD4^+$:$CD8^+$ T-cell ratio during the follow-up period, which suggests that total $CD4^+$ T-cells may be more resistant to the effects of antitumor treatment, in comparison to the total $CD8^+$ T-cells.

Dederichsen and colleagues (2003) reported a significant inverse relationship between the intratumoral $CD4^+$:$CD8^+$ T-cell ratio and survival in patients presenting with colorectal cancer. The implications of this ratio in the peripheral blood has not yet been determined; however, a higher $CD4^+$:$CD8^+$ T-cell ratio in the peripheral blood suggests a weaker $CD8^+$ effector arm of the antitumor immune system, which should in turn be associated with a poorer antitumor immunity. $CD8^+$ T-lymphocytes are directly involved in cancer eradication (Akhmetzyanova et al. 2013). Furthermore, $CD4^+$ T-cells have not been shown to fully compensate for the effector functions of $CD8^+$ T-cells in the absence of $CD8^+$ T-lymphocytes, thus a decline in the $CD8^+$ T-cell subset as a consequence of antitumor treatment may result in a compromised antitumor immunity, and prevent tumor eradication (Akhmetzyanova et al. 2013).

The overall treatment effect showed a declining trend in both the $CD4^+$Treg:total $CD4^+$ T-cell ratio and the $CD8^+$Treg:total $CD8^+$ T-cell ratio, which suggests that despite the fact that antitumor treatment induces a decline in both the Treg and non-Treg components of the immune system, Treg cells are more sensitive to the effects of anticancer treatment, resulting

in a higher relative decline during the follow up period as compared to non-Treg cells. This finding confirms reports by Schmidt and colleagues (2000), who found that the percentage of $CD4^+$ Treg cells in colorectal and breast cancer patients declined distinctly following radiochemotherapy, while the percentage of $CD4^+$ T-lymphocytes remained relatively constant. This finding suggests that the overall effect of anticancer treatment results in an improved antitumor immune profile.

Campbell and colleagues (1973) suggested that all antitumor treatment induces a state of lymphopenia, however in the cohort of patients, described in this study it was demonstrated that the T-cell response to anticancer treatment is heterogeneous and varies depending on the type of malignancy, the clinical stage of disease and the type of antitumor treatment that the patient receives.

Breast cancer patients who received surgical intervention demonstrated a significant variation in the total $CD4^+$ and $CD4^+CD25^+$ T-lymphocyte percentages over the follow-up period. The total $CD8^+$ T-lymphocyte percentage was seen to decline more sharply (-5.62%) in comparison to total $CD4^+$ T-lymphocyte (-1.32%). This was associated with a decline in the total $CD8^+$ T-cell count, while the total $CD4^+$ T-cell count increased almost 2 fold over the follow-up period. This finding contradicts those observed by Lissoni and colleagues (2009), who found a significant decline in the total $CD4^+$ T-cell count in the immediate post-operative period. This difference could be linked to the fact that malignancies under investigation by Lissoni and colleagues were gastrointestinal cancers, as opposed to breast cancer. Furthermore, this difference could also be because immune analysis by Lissoni and colleagues was performed at day 5 post-treatment, while analysis in our cohort occurred at day 1 post-treatment.

Interestingly, a decline in the total $CD8^+$ T-lymphocyte percentage and concentration was observed during the follow-up period with a resultant increase trend in the $CD4^+$:$CD8^+$ T-cell ratio. This suggests that $CD8^+$ T-lymphocytes may be more vulnerable to the effects of surgical treatment than $CD4^+$ T-lymphocytes. This finding motivates for previous reports, which suggested that surgical patients are vulnerable to metastatic implantation of disseminated tumors and enhanced tumor growth during the immediate post operative period (Colachio et al. 1994).

Chemotherapy was initially associated with an increase in the majority of non-Treg cell subsets (%) at day 1 post-treatment, even exceeding levels observed in the surgery group. This was then followed by a substantial decline in both the percentage and count of non-Treg and Treg cell subsets at 5-7 weeks post-treatment, far below levels observed in surgical patients. Similar to findings by Rech and colleagues, breast cancer patients displayed a declining trend in the total $CD4^+$ count, with a $CD4^+$ T-cell count less than 200 cells/µl observed at 5-7 weeks post chemotherapy treatment.

This translated to a substantial decline in the $CD4^+$:$CD8^+$ T-cell ratio from 2 to 0.7 at 5-7 weeks post-treatment, suggesting a substantially compromised antitumor immune profile. This finding illustrates the severe lymphopenia induced by chemotherapy, which has been identified as an independent prognostic factor in patients presenting with colorectal cancer (Chu-yuan et al. 2013). Furthermore, this finding re-emphasizes the importance of assessing an oncology patients' immune profile before the commencement of treatment (Chu-yuan et al. 2013).

As surgery and chemotherapy are generally used in conjunction during the management of breast cancer, it can be assumed that the dual effect of these anticancer modalities on the T-cell immune profile may compromise the patients' immune status to a point that renders them susceptible to opportunistic infections, as seen in patients presenting with Acquired Immune Deficiency Syndrome (AIDS). Therefore, mechanisms need to be employed to optimise oncology patients before treatment and eliminate alternative causes of lymphopenia e.g malnutrition, prior to commencement of treatment (Fock et al. 2010). Secondly, prophylactic treatment against opportunistic infection, similar to those observed in other immune compromised states, should be considered when patients are receiving both surgical and chemotherapeutic treatment for cancer.

Surgical patients demonstrated a substantial decline in the circulating percentage and cell concentration of all Treg cells following exposure to surgical treatment, with the decline in the circulating $CD8^{+}CD25^{+}CD127^{+}$ and $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cells subsets reaching significance. This confirms findings by Tokuno and colleagues (2009), Kono and colleagues (2006), Sellito and colleagues (2011), who found that surgical resection of the tumor results in a significant decline in Treg cells. Some studies have even reported a decline to levels observed in healthy volunteers if all macroscopic tumor was removed (Tokuno et al. 2009, Sellito et al. 2011). This decline could be due to redistribution of Treg cells from the peripheral blood to the site of surgical inflammation (Saito et al. 2013), or it could be that the tumor contains a large induced and expanded Treg pool, which is removed during surgical resection (Tokuno et al. 2009).

All patients who were recipients of chemotherapeutic treatment received a cyclophosphamide-base treatment. This has been shown to inhibit the generation and function

of $CD4^+$ and $CD8^+$ Treg cells, and induce apoptosis in circulating Treg cells (Traverso et al. 2012). Similar to previous reports (Traverso et al. 2012, Rech et al. 2010), the use of cyclophosphamide-based chemotherapy in this cohort was associated with a substantial decline in the circulating $CD4^+$ and $CD8^+$ Treg cell subsets at day 1 post-treatment and at 5-7 weeks post-treatment. Furthermore, although patients received cyclophosphamide in combination with adriamycin 5FU and methotrexate, the latter two treatments have been shown to have a limited effect on the circulating levels of Treg cells (Calasan et al. 2012). In comparison to surgical treatment, chemotherapy induced a more substantial decline in all Treg cell subsets, with surgical patients demonstrating higher Treg levels (%) at 5-7 weeks post-treatment. This could be because chemotherapy has been shown to not only induce apoptosis of Treg cells, but also inhibits $FoxP3^+$ expression at transcriptional level (Xu et al. 2011). As $FoxP3^+$ has been shown as a key regulator in the function of Treg cells, the decrease expression of mRNA inhibits the proliferation of Treg cells in the peripheral blood with a consequent decline in the circulating Treg cell population in the blood (Xu et al. 2011).

Interestingly, despite differences observed in the circulating Treg cell percentages, the Treg cell concentrations was similar in patients who received surgical or chemotherapeutic treatment (Table 7.2). This verifies the findings by Rech and colleagues who reported no difference in the $CD4^+FoxP3^+$ Treg count in early or progressive breast cancer patients who had received surgical or chemotherapeutic anticancer treatment (Rech et al. 2000). Furthermore, they found that the patients presenting with breast cancer displayed similar levels of circulating Treg concentrations to healthy controls (Rech et al. 2000). This may be the result of homeostatic control mechanisms governing the concentration of Treg cell subsets within the peripheral compartment irrespective of the stage of disease or the treatment status (Rech et al. 2010, Parker et al. 2012).

Interestingly, the $CD4^+$ Treg: total $CD4^+$ T-cell ratio in surgical patients decreased during the follow-up period while an upward trend was observed in chemotherapy patients. Furthermore, the $CD8^+$Treg: total $CD8^+$ T-cell ratio was seen to decline at day 1 post-treatment in both surgical and chemotherapy patients. This trend was maintained in surgical patients with a further decline at 5-7 weeks follow-up; however, chemotherapy patients showed an increasing trend from day 1 post-treatment to 5-7 weeks post-treatment. This suggests that $CD4^+$ and $CD8^+$ Treg cells are more susceptible to the effects of surgical intervention, as compared to non-Treg cells. Furthermore, it also suggests that although chemotherapy induces a severe lymphopenia in all T-cell subsets, total $CD4^+$ T-cells are more susceptible to this effect as compared to $CD4^+$ Treg cells, thus resulting in an overall increase in the $CD4^+$ Treg: total $CD4^+$ T-cell ratio. This finding contradicts reports by Lutsiak and colleagues (2005) who found that $CD4^+$ Treg cells are more susceptible to apoptosis following antitumor treatment with chemotherapy, as compared to non-Treg cell subsets. This difference may be because Lutsiak and colleagues (2005) reported findings from *in vitro* and *in vivo* experiments using mouse models. This finding suggests that the surgical removal of the tumor burden is associated with an increase in the relative proportion of antitumor effector $CD4^+$ and $CD8^+$ T-cells, which would translate to a decline in malignancy and Treg mediated immune suppression and improved antitumor response.

The decline observed in the $CD8^+$ Treg: total $CD8^+$ T-cell ratio at day 1 post chemotherapy, and subsequent increase in this ratio at 5-7 weeks post-treatment suggests that $CD8^+$ Treg cells are transiently susceptible to the effects of chemotherapy treatment and their recovery rate post-treatment occurs earlier and at a faster pace than in the total $CD8^+$ T-cell population. This confirms findings by Lutsiak and colleagues (2005) who demonstrate that

cyclophosphamide induces a temporary inhibitory effect on Treg cell populations in the immediate post-treatment period, however Treg cells regain their suppressive function at 10 days post-treatment.

This however does not explain why a similar pattern is not observed in the $CD4^+$ Treg population, suggesting that $CD4^+$ and $CD8^+$ Treg cell populations may have intrinsic differences which results in different responses to the same antitumor treatment.

The increase in the $CD4^+$ Treg: total $CD4^+$ T-cell ratio and $CD8^+$Treg: total $CD8^+$ T-cell ratio following chemotherapy treatment, may direct the immune system towards a more immunosuppressive milieu, which would inhibit active antitumor effector cells as well as, facilitate the conversion of naïve T-lymphocytes to Treg cells thus resulting in the further loss of immune surveillance, tumor progression and even cancer relapse following treatment (Kono et al. 2006).

Due to the late stage of presentation, the majority of patients presenting with oesophageal cancer received palliative radiotherapy with only 20% of patients receiving surgical intervention. A decline was observed in the percentage and count of all $CD4^+$ non-Treg cell subsets; however, despite substantial fluctuation in the different $CD8^+$ T-cells subsets during the follow-up period, a decline was observed in in $CD8^+$ non-Treg T-cell percentage and concentration during the follow-up period. As this downward trend was observed in this cohort despite the majority receiving palliative treatment, it serves as a testament to the aggressive nature of oesophageal cancer and the consequent immune suppression associated with this condition.

Oesophageal cancer patients demonstrated a decline at day 1 post-treatment, which was then followed by an increase in Treg cells at 5-7 weeks post-treatment. Kono and colleagues

(2006) reported that, following the surgically induced decline in Treg cells, a rebound increase in the Treg cell level was observed in patients who presented with disease relapse (Kono et al. 2006). Furthermore, Sellito and colleagues (2011) found that patients who had received palliative surgical excision, and still had remnant cancer or metastatic spread still displayed higher Treg levels than those observed in healthy control patients. (Sellito et al. 2011) This again suggests an association between the tumor burden and circulating Treg levels, with the tumor cells possibly contributing to the underlying mechanism involved in the maintenance and regulation of Treg cells (Tokuno et al. 2009, Sellito et al. 2011). This suggests that Treg cells may potentially be useful as a marker of treatment-response and disease relapse.

Interestingly, an increase was observed in the median percentage of all $CD4^+$ and $CD8^+$ Treg cells over the follow-up period, peaking at 5-7 weeks post-treatment. Curiel and colleagues (2004) demonstrated that $CD4^+CD25^+FoxP3^+$ Treg cells accumulated during tumor progression in patients presented with ovarian cancer, and that these Treg cells suppressed antitumor immunity, and promoted tumor growth (Curiel et al. 2004). In addition to increasing $CD4^+CD25^+FoxP3^+$ Treg cells, oesophageal cancer patients in our cohort demonstrated increasing levels of $CD8^+CD25^+FoxP3^+$ Treg cells, $CD4^+CD127^+FoxP3^+$ Treg, $CD8^+CD127^+FoxP3^+$ Treg, $CD4^+CD25^+CD127^+$ Treg and $CD8^+CD25^+CD127^+$ Treg cells. Breast cancer patients who received chemotherapy or surgical intervention, with curative intent, displayed a decrease in Treg cells over time, while oesophageal cancer patients, the majority (80%) of which received palliative radiotherapy, showed an increasing trend in the Treg profile. This may suggest that either Treg cells may not be susceptible to radio-therapeutic intervention, or the low dose employed during palliative treatment, rather than curative radiotherapy, is insufficient to reduce the circulating levels of Treg cells. However,

the increasing Treg levels in oesophageal cancer patients could also be interpreted as marker of tumor progression, as well as a concomitant predictive marker of response to antitumor treatment.

This increase in Treg cells juxtaposed to the decreasing non-Treg cell profile translated to an increase in the $CD4^+$ Treg: total $CD4^+$ T-cell ratio and $CD8^+$ Treg: total $CD8^+$ T-cell ratio during the follow-up period which suggests a declining antitumor immune response and loss of immune surveillance.

Interestingly, breast cancer patients receiving chemotherapy and oesophageal cancer patients displayed substantially higher levels of $FoxP3^+$ Treg cells (%) in comparison to breast cancer patients who received surgical intervention. Oesophageal cancer patients displayed higher levels of $CD8^+CD25^+FoxP3^+$ Treg cells (%), as compared to all breast cancer patients at all time points assessed, while chemotherapy patients displayed the highest percentage of $CD4^+CD25^+FoxP3^+$ Treg cells at all times assessed. Surgical patients expressed very little $FoxP3^+$ Treg cells. As patients with oesophageal cancer and those requiring chemotherapy treatment presented at a more advanced stage of disease as compared to patients who received surgical intervention, these findings suggest that the frequency of $FoxP3^+$ expressing Treg cells may be associated with the clinical stage of disease at presentation. This corroborates with findings by Sellito and colleagues (2011) who found that higher peripheral Treg levels were associated with advanced stage of colorectal cancer and the presence of distant metastases. Furthermore, Tokuna and colleagues (2009) also reported a significant increase in the frequency of $CD4^+FoxP3^+$ Treg cells in patients with stage 4 gastrointestinal cancer, including oesophageal cancer, as compared to patients with stage I, II and III gastrointestinal cancer or healthy controls. This may suggest that although, as previously

reported, an increased frequency of Treg cells is observed in patients presenting with malignancy, late stage of disease may be associated with an increased frequency of Treg cells expressing FoxP3 receptor.

Although this study demonstrated the alterations which occur in the peripheral system in response to anticancer treatment, this may not be a true reflection of the physiology of non-Treg and Treg cells at the tumor microenvironment. Thus future studies would aim to analyse the T-lymphocyte levels in both the peripheral blood and tumor microenvironment, thus revealing the true

8 Treatment outcome and association with T-lymphocyte immune profile

8.1 Introduction

Predictive and prognostic markers in cancer play an important role in the decision making process during the management of patients. Despite the identification and use of a number of prognostic and predictive factors in breast and oesophageal cancer, the search for a feasible and more reliable marker is still ongoing. Following studies that demonstrated the crucial contribution that the host immune system makes towards tumor eradication, research efforts have been directed towards the identification of immunological markers which can be used in clinical practice. This marker would facilitate the identification of patients who would benefit from treatment and those at high risk of mortality, as well as to monitor the progress of patients receiving treatment.

Numerous studies have evaluated the relationship between $CD4^+$, $CD8^+$ and Treg T-lymphocyte distribution and patient outcome, however this has yielded conflicting results (Alvaro et al. 2005, Bates et al. 2006, Ladoire et al. 2008, Aruga et al. 2009, Salama et al. 2009, Haas et al. 2009, Correale et al. 2010). This study aimed to determine the relationship between T-lymphocyte subsets before and after treatment compared to patient outcome at 5-7 week follow-up. The outcomes of interest included survival or demise before the 5-7 week follow-up period. Statistical analysis was conducted using the unpaired non-parametric Mann-Whitney statistic with a two-sided P-value. A P-value of <0.05 was considered as statistically significant.

8.2 Results

8.2.1 Detection of $CD4^+$ and $CD8^+$ T-lymphocyte populations in patients who survived compared to those who demised during the 5-7 week follow-up period

Patients who demised were found to have substantially lower $CD4^+$ T-lymphocyte percentage (Figure 8.1) and cell concentration (cells/µl) (Table 8.1) before treatment and at day 1 post-treatment, as compared to patients who survived; with a significant difference observed in the total $CD4^+$ T-cell percentage (P=0.034) before treatment, as well as the $CD4^+CD25^-$ T-cell percentage (P=0.038) and total $CD4^+$ T-lymphocyte concentration (P=0.0046) at day 1 post-treatment (Figure 8.1 and Table 8.1). Furthermore, in comparison to patients who survived, patients who demised where also found to have a higher circulating percentage of $CD8^+$ non-Treg T-lymphocyte subsets, with a significant difference observed in the median percentage of $CD8^+CD45^+$ T-lymphocyte (P=0.01) and $CD8^+CD25^-$ T-lymphocytes (P=0.013) (Figure 8.1). This however did not translate to a higher $CD8^+$ T-cell concentration, with patients who demised found to have a lower $CD8^+$ T-lymphocyte concentration before receiving anti-tumor treatment (190 cells/µl versus. 140 cells/µl; P=0.79) and at day 1 post-treatment (160 cells/µl versus. 88 cells/µl; P=0.058) (Table 8.1). This translated to a $CD4^+$: $CD8^+$ T-cell ratio of 1.2 in patients who demised compared to 2.2 in patients who survived, this was statistically significant (P=0.044) (Table 8.1). The $CD4^+$: $CD8^+$ T-cell ratio was seen to improve in patients who survived at day1 after treatment ($CD4^+$: $CD8^+$ T-cell ratio = 2.5), however this ratio remained stable in patients who demised ($CD4^+$: $CD8^+$ T-cell ratio = 1.2) (Table 8.1).

Table 8.1: The total $CD4^+$ T-lymphocyte count, total $CD8^+$ T-lymphocyte count, and $CD4^+$: $CD8^+$ T-cell ratio in breast and oesophageal cancer patients who survived and demised following antitumor treatment.

	Survived	**Demised**	**Statistical significance (P)**
Total $CD4^+$ count (cells/µl)			
Before treatment	345 (IQR: 172-558)	205 (IQR: 147-316)	0.137
After treatment	390 (IQR: 230-472)	111 (IQR: 101-153)	0.0046**
Total $CD8^+$ count (cells/µl)			
Before treatment	190 (IQR: 61-253)	140 (IQR: 79-252)	0.790
After treatment	160 (IQR: 91-214)	88 (IQR: 64-112)	0.058*
$CD4^+$: $CD8^+$ T-cell ratio			
Before treatment	2.2 (IQR: 1.5-3.5)	1.2 (IQR: 1.1-1.9)	0.044*
Day 1 after treatment	2.5 (IQR: 1.6-3.4)	1.2 (IQR: 0.9-2.4)	0.186
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)			

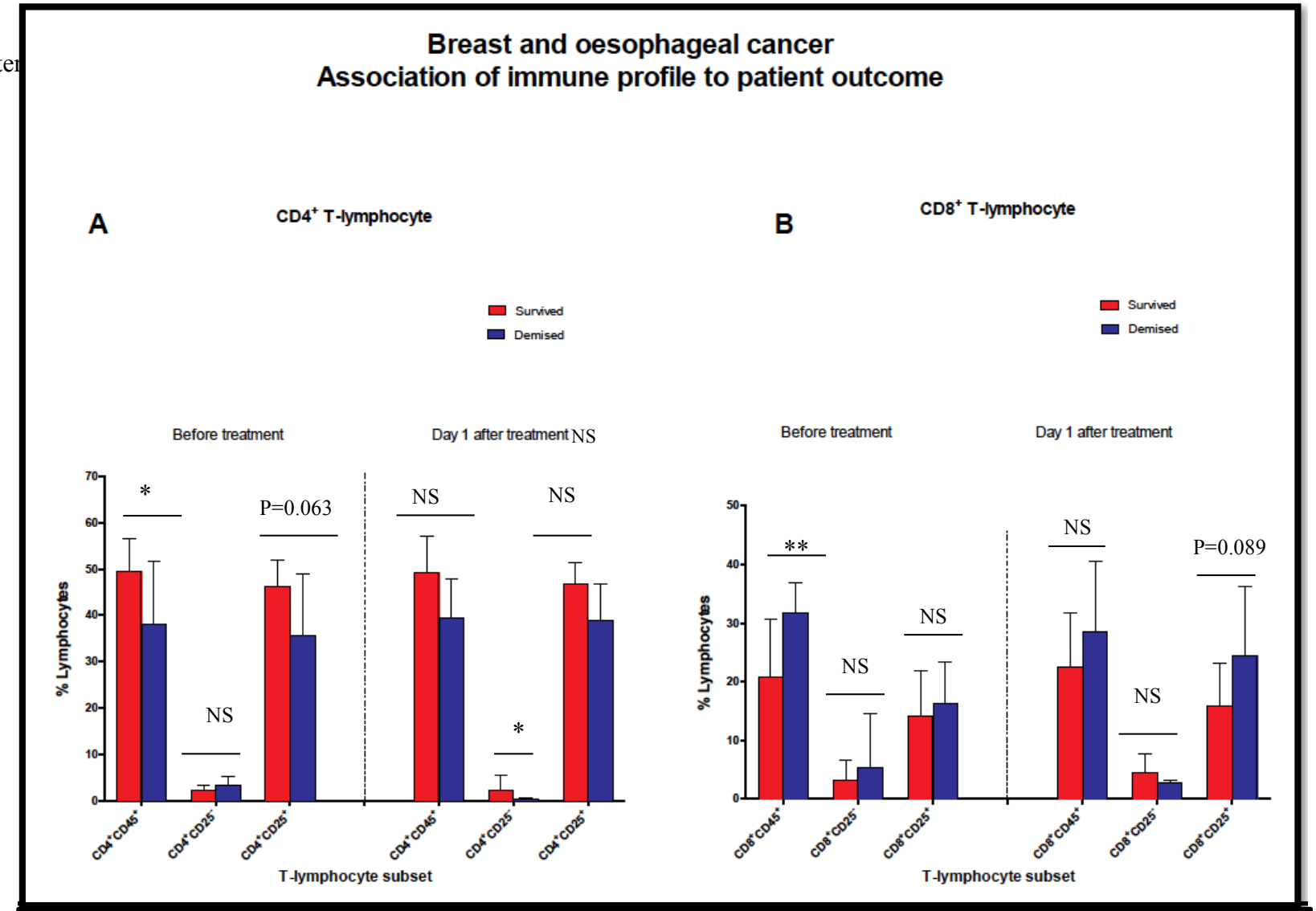


Figure 8.1: **The frequency distribution of $CD4^+$ and $CD8^+$ T-lymphocyte subsets of breast and oesophageal cancer in patients who survived (red bar) and demised (red bar), before treatment and at day 1 post-treatment**. This is shown as the median percentage (%) ±IQR. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

8.2.2 Detection of $CD4^+$ and $CD8^+$ Treg cell populations in patients who survived compared to those who demised during the 5-7 week follow-up period

Patients who survived displayed a similar Treg percentage and count as compared to patients who demised. However, a significantly higher prevalence of $CD8^+CD25^+CD127^+$ Treg cells (%) was observed before treatment in patients who survived, as opposed to patients who demised (P=0.019) (Figure 8.2). Furthermore, the $CD8^+CD25^+CD127^+$ Treg concentration in patients who survived was higher as compared to those who demised before treatment and at day 1 post-treatment, however this did not reach significance (Table 8.2). This translated to a significantly higher $CD8^+$ Treg: total $CD8^+$ T-cell ratio (P=0.0126), before treatment, in patients who survived compared to patients who demised (Table 8.2 and Table 8.3).

Table 8.2: The $CD4^+$ and $CD8^+$ Treg cell count (cells/µl) in breast and oesophageal cancer patients who survived and demised, before receiving antitumor treatment and at day 1 post-treatment.

	Survived	Demised	Statistical significance (P)
Before treatment			
$CD4^+$ Treg cells (cells/µl)			
$CD4^+CD25^+FoxP3^+$	0 (IQR: 0-0)	0 (IQR: 0-0)	0.89
$CD4^+CD127^+FoxP3^+$	0 (IQR: 0-0)	0 (IQR: 0-0)	0.311
$CD4^+CD25^+CD127^+$	10 (IQR: 2.8-21.9)	9 (IQR: 4-11)	0.473
$CD8^+$ Treg cells (cells/µl)			
$CD8^+CD25^+FoxP3^+$	0 (IQR: 0-0.3)	0 (IQR: 0-0)	0.6
$CD8^+CD127^+FoxP3^+$	0 (IQR: 0-0)	0 (IQR: 0-3)	0.362
$CD8^+CD25^+CD127^+$	46 (IQR: 18-72)	21 (IQR: 11-38)	0.137
Day 1 after treatment			
$CD4^+$ Treg cells (cells/µl)			
$CD4^+CD25^+FoxP3^+$	0 (IQR: 0-1)	0 (IQR: 0-0)	0.395
$CD4^+CD127^+FoxP3^+$	0 (IQR: 0-0)	0 (IQR: 0-0)	0.629
$CD4^+CD25^+CD127^+$	9 (IQR: 4-17)	2 (IQR: 2-4)	0.047*
$CD8^+$ Treg cells (cells/µl)			
$CD8^+CD25^+FoxP3^+$	0 (IQR: 0-0)	0 (IQR: 0-4)	0.113
$CD8^+CD127^+FoxP3^+$	0 (IQR: 0-0)	0 (IQR: 0-0)	0.942
$CD8^+CD25^+CD127^+$	27 (IQR: 16-42)	15 (IQR: 12-22)	0.237
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)			

Table 8.3: The $CD4^+$ Treg (%): total $CD4^+$ (%) and $CD8^+$ Treg (%): total $CD8^+$ (%) T-cell ratios in breast and oesophageal cancer patients who survived and demised, before treatment and at day 1 after treatment

$CD4^+$Treg: total $CD4^+$ T-cell ratio and $CD8^+$Treg: total CD^+ T-cell ratio in breast and oesophageal cancer patients			
	Survived	**Demised**	**Statistical significance (P)**
$CD4^+$ Treg: total $CD4^+$ T-cell ratio			
Before treatment	0.06 (IQR: 0.02-0.11)	0.08 (IQR: 0.03-0.13)	0.7101
Day 1 after treatment	0.04 (IQR: 0.03-0.11	0.06 (IQR: 0.04-0.73)	0.924
$CD8^+$ Treg: total $CD8^+$ T-cell ratio			
Before treatment	1.48 (0.69-1.98)	0.53 (0.26-0.91)	0.012**
Day 1 after treatment	0.84 (IQR: 0.53-1.33)	0.87 (IQR: 0.58-1.00)	0.7768
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)			

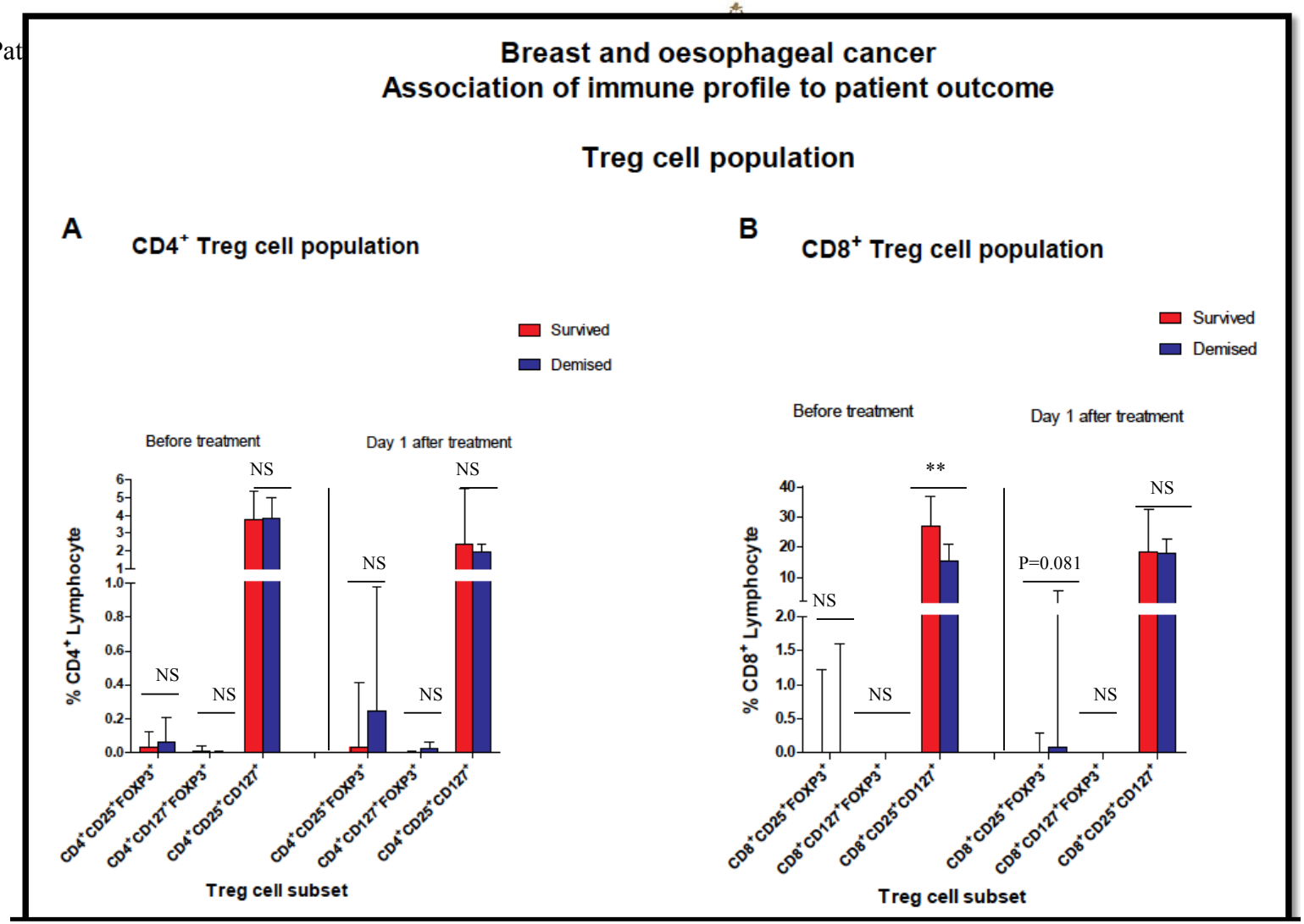


Figure 8.2: The frequency distribution of $CD4^+$ and $CD8^+$ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment. This is shown as the median percentage (%) ±IQR. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

8.2.3 Detection of $CD4^+$ and $CD8^+$ Treg cell populations in patients who survived compared to those who demised during the 5-7 week follow-up period, stratified according to tumor type

8.2.3.1 $CD4^+$ Treg cell population

Patients presenting with oesophageal cancer displayed a higher frequency of FoxP3-expressing Treg cells as compared to patients presenting with breast cancer (Figure 8.3). Due to the small sample size in each cancer type, statistical analysis could not be conducted.

8.2.3.2 $CD8^+$ Treg cell population

Patients presenting with breast and oesophageal cancer displayed similar levels of $CD8^+$ FoxP-3 expressing and non-FoxP3 Treg cells (Figure 8.4). Due to the small sample size in each cancer type, statistical analysis could not be conducted.

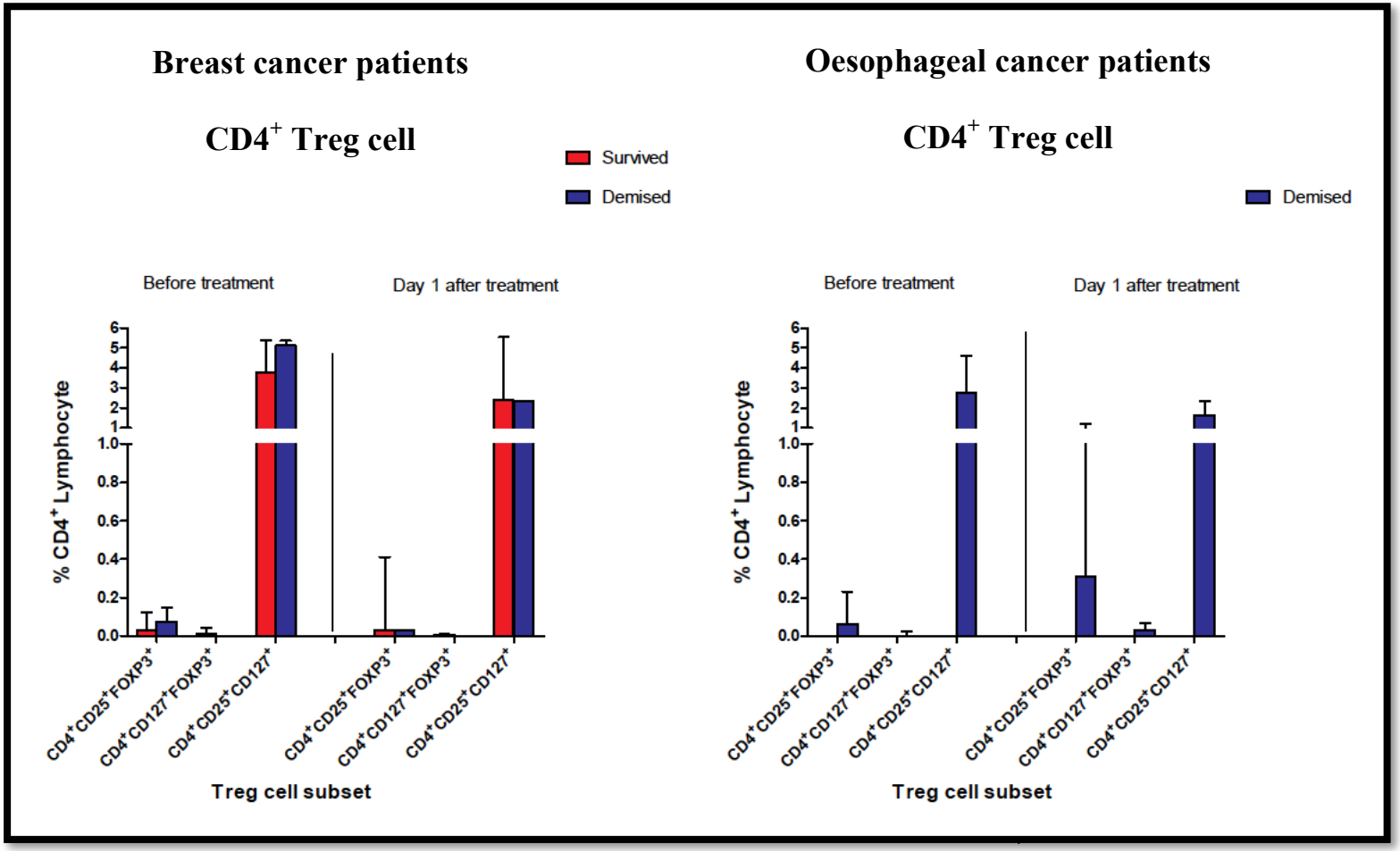


Figure 8.3:The frequency distribution of CD4$^+$ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment. This is shown as the median percentage (%) ±IQR. ((NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

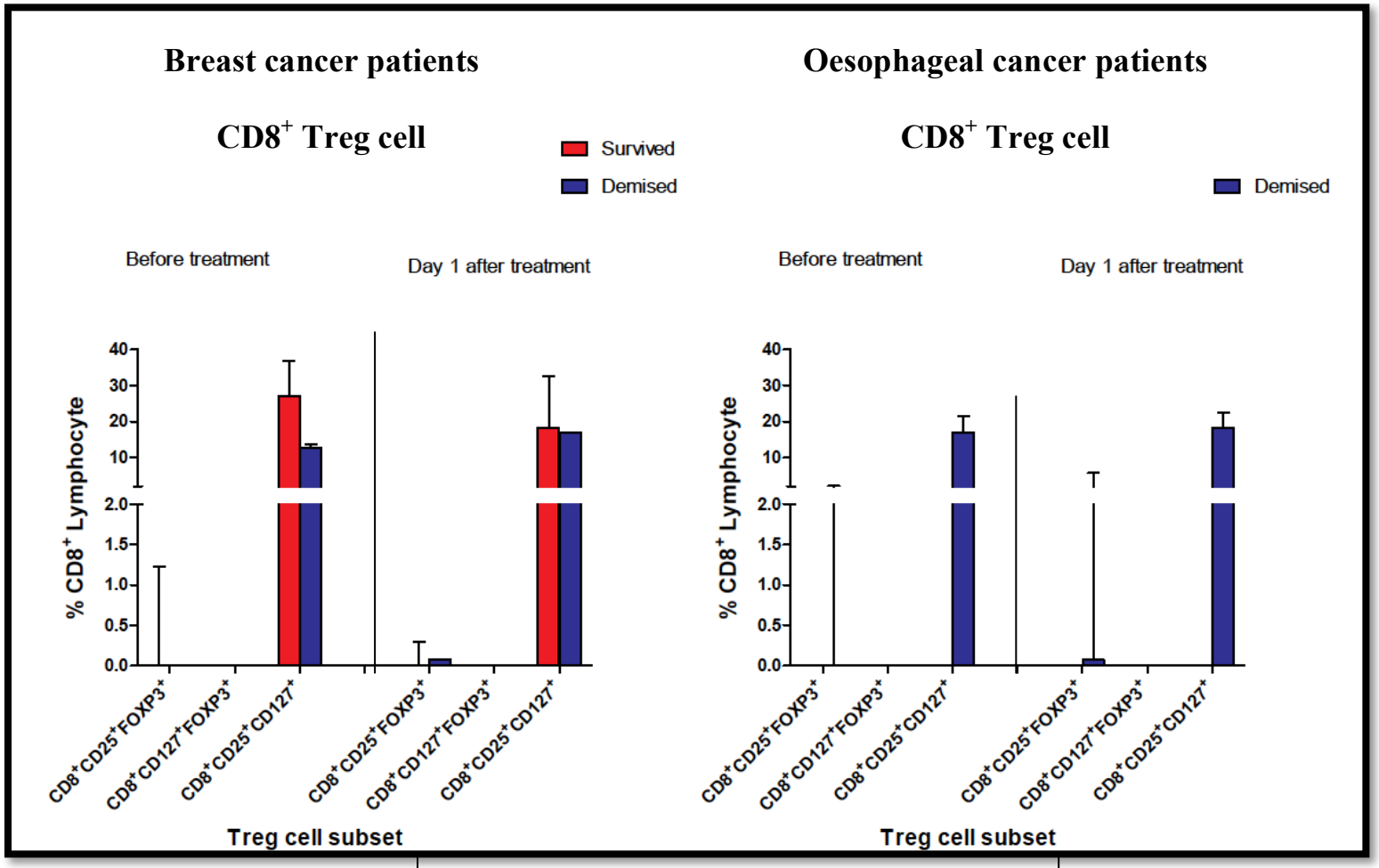


Figure 8.4: The frequency distribution of CD8$^+$ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment. This is shown as the median percentage (%) ±IQR. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

8.3 Discussion

$CD4^+$, $CD8^+$ and Treg T-lymphocytes have all been implicated as possible predictors of prognosis, and for monitoring of treatment response. Numerous studies have reported that the increased levels of tumor infiltrating and circulating $CD4^+$ and $CD8^+$ T-lymphocytes correlate with a good prognosis in the context of cancer (Ayoub et al 1998, Zingg et al. 2010), while increased levels of Treg cells in the peripheral blood and tumor microenvironment has been associated with a poor prognosis. (Curiel et al. 2004, Bates et al. 2006, Wolf et al. 2007).

In this cohort, it was demonstrated that patients who demised presented with a lower prevalence of $CD4^+$ T-lymphocyte percentage and concentration, which supports the findings by Ray-Coquad and colleagues (2009) who reported that $CD4^+$ lymphopenia is associated with poor outcome. Furthermore, patients who survived displayed a significantly higher $CD8^+$ T-lymphocyte count at all time points, as compared to patients who demised. This confirms findings by Blake-more and colleagues (2010), who reported that a higher $CD8^+$ T-lymphocyte count was associated with longer survival in patients presenting with breast cancer, independent of the effects of medical treatment (Blake-more et al. 2010).

A $CD4^+$: $CD8^+$ T-cell ratio of 2.1 was found in patients who survived, compared to a ratio of 1.2 in those who demised. This verifies findings by Diederichsen and colleagues (2003) who demonstrated that patients suffering from colorectal cancer, with a good clinical outcome and higher 5-year survival displayed a $CD4^+$:$CD8^+$ T-lymphocyte ratio of > 2. However, they

assessed this ratio in tumor infiltrating lymphocytes, while this study assessed this ratio in the peripheral blood.

Despite numerous reports suggesting that a higher Treg cell level in the peripheral blood is associated with a poor prognosis (Kono et al. 2006), this was not observed in this cohort. On the contrary, patients who survived displayed higher levels of $CD8^+CD25^+CD127^+$ Treg cell percentage and cell concentration before treatment and at day 1 post-treatment, which translated to a significantly higher $CD8^+$Treg: total $CD8^+$ T-cell ratio, as compared to patients who demised. Furthermore, the $CD4^+CD25^+CD127^+$ Treg cell count in patients who survived was significantly higher than that observed in patients who demised. This suggests that FoxP3-negative Treg cells may be associated with improved outcome, which contradicts findings by Liu and colleagues (2008) who reported a poor prognosis in cancer patients who displayed a high percentage of $CD4^+CD25^+$ Treg cells.

Interestingly, patients who survived displayed an absence of FoxP3-positive Treg cells, while patients who demised, most notably patients presenting with oesophageal cancer, displayed a higher prevalence of $FoxP3^+$ Treg cell. This correlates with findings by Yamamato and colleagues (2012), who reported that pancreatic cancer patients presenting with a low circulating percentage of $FoxP3^+$ Treg cells displayed a longer period of survival as compared to patients with higher levels of $FoxP3^+$ Treg cells. However, FoxP3-negative T-lymphocytes were not evaluated by Yamamato and colleagues (2012).

When patients were stratified according to the type of cancer, oesophageal cancer patients displayed higher levels of circulating $FoxP3^+$ Treg cells, as compared to breast cancer patients who survived and demised. This suggests that the increase in $FoxP3^+$ expression may be tumor specific, which contradicts other studies which have identified $FoxP3^+$ Treg cells in

peripheral circulatory system and within the tumor microenvironment in patients presenting with breast cancer (Ladoire et al .2008, Perez et al. 2007). The expression of $FoxP3^+$ could also be as a result of the aggressive nature of the malignancy, the late stage of presentation of all oesophageal cancer patients, the severe immune deficiency as a consequence of both the nutritional depletion and the progressive malignancy. The absence of oesophageal patients who survived until 5-7 weeks follow-up prevents further investigation of this point. Future studies, with larger sample sizes would enable us to investigate which factors in oesophageal cancer patients contribute to the induction of $FoxP3^+$ expression. The clear differences observed between $FoxP3^+$ and $FoxP3^-$ Treg cells in this cohort, implies that therapeutic treatments aimed at the eradication of all Treg cells in oncology patients may have beneficial effects, but may also result in the depletion of T-cell subsets which are involved either directly or indirectly in the improvement of patient outcome. This is a contentious issue considering reports that the effect which Treg cells confers their effect in the host is heterogeneous and may be organ or tumor specific (Zingg et al. 2010). More efforts need to be employed to further discern the role of these cells in malignancy, the clinical implications of Fox-P3 expression versus non-FoxP3 Treg cells and how best to manipulate these cells so as to promote improved patient outcome.

9 Conclusion

Within the context of cancer, the immune system has been shown to have two distinct arms which play opposing roles. These include immune cells which facilitate and augment tumor growth and progression, while the other component attenuates tumor progression and eradicates existing malignant cells. The conundrum in the management of malignancy lies in the question of how best to up-regulate the antitumor arm of the immune system and simultaneously attenuate the pro-tumor component. The pivotal regulatory role that the immune system plays in the development and progression of cancer suggests that elements of this complex system may vary depending on the type, size, location and aggressiveness of a tumor. This implies that changes to the tumor as a consequence of antitumor treatment, would also result in a change in the patients' immune status. Taken together this suggests that the patients' immune status may be the predictive and prognostic marker which the world has been searching for. Despite the limitations of this study, there is strong evidence to suggest that the immune profile of a cancer patient should be taken into consideration when deciding on the mode and aggressiveness of treatment, as well as when estimating patient survival.

10 References

Abbas, A., Lichtman, A. and Pillai, S., (2007). Innate and adaptive immunity.C*ellular and Molecular Immunology*. 6 edn. Philadelphia, USA: Saunders Elsevier, pp. 4-17.

Abbas, A., Lichtman, A. and Pillai, S., (2007b). Lymphocyte development and the rearrangement and expression of antigen receptor genes. *Cellular and Molecular Immunology*. 6 edn. Philadelphia, USA: Saunders Elsevier, pp. 153-187.

Abbas, A., Lichtman, A. and Pillai, S., (2007c). Immunity to tumors. *Cellular and Molecular Immunology*. 6 edn. Philadelphia, USA: Saunders Elsevier, pp. 397-417.

Adami, J., Gäbel, H., Lindelöf, B., Ekström, K, Rydh, B., Glimelius, B., Ekbom, A., Adami, H. and Granath, F., (2003). Cancer risk following organ transplantation: A nationwide cohort study in Sweden.B*ritish Journal of Cancer,* **89**(7), pp. 1221-1227.

African Cancer Registry Network, (2014). National cancer registry of South Africa. [Online] Available from: http://afcrn.org/membership/membership-list/87-ncrsa (Accessed: 23/03/2014).

Agarwala, S.S. and Ribas, A., (2010). Current experience with CTLA-4-blocking monoclonal antibodies for the treatment of solid tumors. *Journal of Immunotherapy (Hagerstown, md.: 1997),* **33**(6), pp. 557-569.

Akamatsu, M., Matsumoto, T., Oka, K., Yamasaki, S., Sonoue, H., Kajiyama, Y., Tsurumaru, M. and Sasai, K., (2003). C-erbb-2 oncoprotein expression related to chemoradioresistance in esophageal squamous cell carcinoma. *International Journal of Radiation Oncology* Biology* Physics,* **57**(5), pp. 1323-1327.

Akhmetzyanova, I., Zelinskyy, G., Schimmer, S., Brandau, S., Altenhoff, P., Sparwasser, T. and Dittmer, U., (2013). Tumor-specific $CD4^+$ T-cells develop cytotoxic activity and eliminate virus-induced tumor cells in the absence of Regulatory T-cells. *Cancer Immunology, Immunotherapy,* **62**(2), pp. 257-271.

Alcaraz, A., González-López, R., Morote, J., De la Piedra, C., Meseguer, C., Esteban, E., Climent, M., González-Gragera, B., Alvarez-ossorio, J. and Chirivella, I., (2013). Biochemical markers of bone turnover and clinical outcome in patients with renal cell and bladder carcinoma with bone metastases following treatment with Zoledronic acid: the Tugamo study. *British Journal of Cancer,* **109**(1), pp. 121-130.

Allan, S.E., Crome, S.Q., Crellin, N.K., Passerini, L., Steiner, T.S., Bacchetta, R., Roncarolo, M.G. and Levings, M.K., (2007). Activation-induced FoxP3 in human T-effector cells does not suppress proliferation or cytokine production. *International Immunology,* **19**(4), pp. 345-354.

Allred, D.C., Clark, G.M., Molina, R., Tandon, A.K., Schnitt, S.J., Gilchrist, K.W., Osborne, C.K., Tormey, D.C. and Mcguire, W.L., (1992). Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. *Human pathology,* **23**(9), pp. 974-979.

Altfeld, M., Addo, M.M., Kreuzer, K.A., Rockstroh, J.K., Dumoulin, F.L., Schliefer, K., Leifeld, L., Sauerbruch, T. and Spengler, U., (2000). T(h)1 to T(h)2 shift of cytokines in peripheral blood of HIV-infected patients is detectable by reverse transcriptase polymerase chain reaction but not by enzyme-linked immunosorbent assay under nonstimulated conditions. *Journal of Acquired Immune Deficiency Syndromes,* **23**(4), pp. 287-294.

Altman, D.G., Lausen, B., Sauerbrei, W. and Schumacher, M., (1994). Dangers of using "optimal" cutpoints in the evaluation of prognostic factors. *Journal of the National Cancer Institute,* **86**(11), pp. 829-835.

Altman, D.G. and Lyman, G.H., (1998). Methodological challenges in the evaluation of prognostic factors in breast cancer. *Breast cancer research and treatment,* **52**(1-3), pp. 289-303.

Alvaro, T., Lejeune, M., Salvado, M.T., Bosch, R., Garcia, J.F., Jaen, J., Banham, A.H., Roncador, G., Montalban, C. and Piris, M.A., (2005). Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and

Regulatory T-cells. *Clinical Cancer Research : An official journal of the American association for cancer research,* **11**(4), pp. 1467-1473.

American cancer society.,(2013) How is cancer of the esophagus staged. [Online] Available from: http://www.cancer.org/cancer/esophaguscancer/detailedguide/esophagus-cancer-staging (Accessed: 22/01/2014).

American Cancer Society., (2011). cancer in africa. *Atlanta: American cancer society,* [Online] Available from: http://www.cancer.org/acs/groups/cid/documents/webcontent/002550-pdf.pdf (Accessed 30/12/2013).

American Cancer Society., (2011) Oncogenes, tumor suppressor genes and cancer. [Online] Available from: http://www.cancer.org/acs/groups/cid/documents/webcontent/002550-pdf.pdf (Accessed 30/12/2013).

Andersen, M.H., Sorensen, R.B., Brimnes, M.K., Svane, I.M., Becker, J.C. and Thor Straten, P., (2009). Identification of heme oxygenase-1-specific Regulatory $CD8^+$ T-cells in cancer patients. *The Journal of Clinical Investigation,* **119**(8), pp. 2245-2256.

Anderson, R., (2011). *Head of the immunology department; University of Pretoria.*

Appay, V., Zaunders, J.J., Papagno, L., Sutton, S., Jaramillo, A., Waters, A., Easterbrook, P., Grey, P., Smith, D. and Mcmichael, A.J., (2002). Characterization of CD4 CTLs ex vivo. *The Journal of Immunology,* **168**(11), pp. 5954-5958.

Aruga, T., Suzuki, E., Saji, S., Horiguchi, S., Horiguchi, K., Sekine, S., Kitagawa, D., Funata, N., Toi, M., Sugihara, K. and Kuroi, K., (2009). A low number of tumor-infiltrating FoxP3-positive cells during primary systemic chemotherapy correlates with favorable anti-tumor response in patients with Breast cancer. *Oncology Reports,* **22**(2), pp. 273-278.

Azuma, T., Takahashi, T., Kunisato, A., Kitamura, T. and Hirai, H., (2003). Human $CD4^+$ $CD25^+$ Regulatory T-cells suppress NKT-cell functions. *Cancer Research,* **63**(15), pp. 4516-4520.

Bachtiary, B., Herbacek, I., Zideck, T., Knocke, T.H., Dimopoulos, J., Poetter, R. and Micksche, M., (2005). Impact of radiotherapy with and without concurrent Cisplatin on lymphocyte subpopulations in cervical cancer patients. *Anticancer Research,* **25**(6c), pp. 4673-4678.

Baratelli, F., Lin, Y., Zhu, L., Yang, S.C., Heuze-Vourc'h, N., Zeng, G., Reckamp, K., Dohadwala, M., Sharma, S. and Dubinett, S.M., (2005). Prostaglandin E2 induces FoxP3 gene expression and T regulatory cell function in human $CD4^+$ T-cells. *Journal of Immunology (Baltimore, MD.: 1950),* **175**(3), pp. 1483-1490.

Barnett, B., Kryczek, I., Cheng, P., Zou, W. and Curiel, T.J., (2005). Regulatory T-cells in Ovarian cancer: biology and therapeutic potential. *American Journal of Reproductive Immunology,* **54**(6), pp. 369-377.

Basu, R., O'Quinn, D.B., Silberger, D.J., Schoeb, T.R., Fouser, L., Ouyang, W., Hatton, R.D. and Weaver, C.T., (2012). Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. *Immunity,**37***(6), pp. 1061-1075

Bates, G.J., Fox, S.B., Han, C., Leek, R.D., Garcia, J.F., Harris, A.L. and Banham, A.H., (2006). Quantification of Regulatory T-cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *Journal of Clinical Oncology,* **24**(34), pp. 5373-5380.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L. and Spies, T., (1999). Activation of NK cells and T-cells by NKG2D, a receptor for stress-inducible mica. *Science,* **285**(5428), pp. 727-729.

Baumgartner, J.M. and Mccarter, M.D., (2009). Suppressing the suppressor: role of immunosuppressive Regulatory T-cells in cancer surgery. *Surgery,* **145**(4), pp. 345-350.

Bellamy, W.T, Richter, L., Sirjani, D., Roxas, C., Glinsmann-Gibson, B., Frutiger, Y., Grogan, T.M. and List, A.F., (2001). Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature Myeloid precursors and Leukemia progenitor formation in Myelodysplastic syndromes. *Blood,* **97**(5), pp. 1427-1434.

Ben‐Eliyahu, S., Page, G.G., Yirmiya, R. and Shakhar, G., (1999). Evidence that stress and surgical interventions promote tumor development by suppressing Natural Killer cell activity. *International Journal of Cancer,* **80**(6), pp. 880-888.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F. and Ochs, H.D., (2001). The Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked Syndrome (IPEX) is caused by mutations of FoxP3. *Nature Genetics,* **27**(1), 20-21.

Bird, P., Hill, A. and Houssami, N., (2008). Poor hormone receptor expression in East African breast cancer: evidence of a biologically different disease? *Annals of Surgical Oncology,* **15**(7), pp. 1983-1988.

Birkeland, S.A., Storm, H.H., Lamm, L.U., Barlow, L., Blohmé, I., Forsberg, B., Eklund, B., Fjeldborg, O., Friedberg, M. and Frödin, L., (1995). Cancer risk after renal transplantation in the Nordic countries, 1964–1986. *International Journal of Cancer,* **60**(2), pp. 183-189.

Blake-mortimer, J.S., Sephton, S.E., Carlson, R.W., Stites, D. and Spiegel, D., (2004). Cytotoxic T-lymphocyte count and survival time in women with metastatic breast cancer. *Breast Journal,* **10**(3), pp. 195-199.

Blank, C., Brown, I., Peterson, A.C., Spiotto, M., Iwai, Y., Honjo, T. and Gajewski, T.F., (2004). PD-l1/b7h-1 inhibits the effector phase of tumor rejection by T-cell receptor (TCR) transgenic $CD8^+$ T-cells. *Cancer Research,* **64**(3), pp. 1140-1145.

Bofill,M., Janossy, G., Lee, C., Macdonald‐Burns, D., Phillips, A., Sabin, C., Timms, A., Johnson, M. and kernoff, p., (1992). Laboratory control values for $CD4^+$

and $CD8^+$ T-lymphocytes. implications for HIV - 1 diagnosis. *Clinical and Experimental Immunology,* **88**(2), pp. 243-252.

Bogaert, L., (2013). Breast cancer molecular subtypes as identified by immunohistochemistry in South African black women. *The Breast Journal,* **19**(2), pp. 210-211.

Bonanno, G., Mariotti, A., Procoli, A., Folgiero, V., Natale, D., De Rosa, L., Majolino, I., Novarese, L., Rocci, A., Gambella, M., Ciciarello, M., Scambia, G., Palumbo, A., Locatelli, F., De Cristofaro, R. and Rutella, S., (2012). Indoleamine 2,3-Dioxygenase 1 (IDO1) activity correlates with immune system abnormalities in Multiple myeloma. *Journal of Translational Medicine,* **10**, pp. 247-5876-10-247.

Bray, F., Jemal, A., Grey, N., Ferlay, J. and Forman, D., (2012). Global cancer transitions according to the human development index (2008–2030): a population-based study. *The Lancet Oncology,* **13**(8), pp. 790-801.

Bremers, A.J. and Parmiani, G., (2000). Immunology and immunotherapy of human cancer: present concepts and clinical developments. *Critical Reviews in Oncology-Hematology,* **34**(1), pp. 1-25.

Bridoux, F., Badou, A., Saoudi, A., Bernard, I., Druet, E., Pasquier, R., Druet, P. and Pelletier, L., (1997). Transforming Growth Factor Beta (TGF-beta)-dependent inhibition of T helper cell 2 (Th2)-induced autoimmunity by self-Major Histocompatibility Complex (MHC) class II-specific, Regulatory $CD4^+$ T-cell lines. *The journal of experimental medicine,* **185**(10), pp. 1769.

Broad Institute of MIT, (2014) Oncogene. [Online] Available from:: http://www.broadinstitute.org/education/glossary/oncogene (Accessed: 10/01/ 2014).

Brodsky, R.A., Chen, A.R., Dorr, D., Fuchs, E.J., Huff, C.A., Luznik, L., Smith, B.D., Matsui, W.H., Goodman, S.N., Ambinder, R.F. and Jones, R.J., (2010). High-dose Cyclophosphamide for severe aplastic anemia: long-term follow-up. *Blood,* **115**(11), pp. 2136-2141.

Bromberg, J.F., Horvath, C.M., Wen, Z., Schreiber, R.D. and Darnell, J.E., (1996). Transcriptionally active STAT1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proceedings of the national academy of sciences,* **93**(15), pp. 7673-7678.

Bronte, V., Chappell, D.B., Apolloni, E., Cabrelle, A., Wang, M., Hwu, P. and Restifo, N.P., (1999). Unopposed production of Granulocyte-Macrophage Colony-Stimulating Factor by tumors inhibits $CD8^+$ T-cell responses by dysregulating antigen-presenting cell maturation. *Journal of Immunology (Baltimore, MD.: 1950),* **162**(10), pp. 5728-5737.

Brown, L.M., Hoover, R., Silverman, D., Baris, D., Hayes, R., Swanson, G.M., Schoenberg, J., Greenberg, R., Liff, J., Schwartz, A., Dosemeci, M., Pottern, L. and Fraumeni, J.F.,jr, (2001). Excess incidence of squamous cell Esophageal cancer among us black men: role of social class and other risk factors. *American Journal of Epidemiology,* **153**(2), pp. 114-122.

Burnet, M., (1957). Cancer—a biological approach: iii. Viruses associated with neoplastic conditions. iv. practical applications. *British Medical Journal,* **1**(5023), pp. 841.

Campbell, A.C., Hersey, P., Harding, B., Hollingsworth, P.M., Skinner, J. and Maclennan, I.C., (1973). Effects of anti-cancer agents on immunological status. *The British Journal of CancerSsupplement,* **1**, pp. 254-261.

CANSA, (2014) South African cancer statistics. [Online] Available from: http://www.cansa.org.za/south-african-cancer-statistics/ (Accessed 23/03/2014).

Carey, T.E., Takahashi, T., Resnick, L.A., Oettgen, H.F. and Old, L.J., (1976). Cell surface antigens of human Malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. *Proceedings of the National Academy of Sciences,* **73**(9), pp. 3278-3282.

Carrock Sewell, W., North, M.E., Webster, A.D.B. and Farrant, J., (1997). Determination of intracellular cytokines by flow-cytometry following whole-blood culture. *Journal of Immunological Methods,* **209**(1), pp. 67-74.

Carter, C., Allen, C. and Henson, D., (1989). Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. *Cancer,* **63**, pp. 181-187.

CDC, (2004) Laboratory procedure manual: $CD4^+$ T-lymphocytes and $CD8^+$ T-cells. [Online] Available from: http://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/l03_c_met_cd4+_cd8+.pdf (Accessed 18/01/2014).

Chen, W., Jin, W., Hardegen, N., Lei, K., Li, L., Marinos, N., Mcgrady, G. and Wahl, S.M., (2003). Conversion of peripheral $CD4^+CD25^-$ naive T-cells to $CD4^+CD25^+$ Regulatory T-cells by TGF-β induction of transcription factor FoxP3. *The Journal of Experimental Medicine,* **198**(12), pp. 1875-1886.

Cho, Y., Miyamoto, M., Kato, K., Fukunaga, A., Shichinohe, T., Kawarada, Y., Hida, Y., Oshikiri, T., Kurokawa, T., Suzuoki, M., Nakakubo, Y., Hiraoka, K., Murakami, S., Shinohara, T., Itoh, T., Okushiba, S., Kondo, S. and Katoh, H., (2003). $CD4^+$ and $CD8^+$ T-cells cooperate to improve prognosis of patients with Esophageal squamous cell carcinoma. *Cancer Research,* **63**(7), pp. 1555-1559.

Christakis, N.A., Lamont, E.B., Smith, J.L. and Parkes, C.M., (2000). Extent and determinants of error in doctors' prognoses in terminally ill patients: prospective cohort studycommentary: why do doctors overestimate? commentary: prognoses should be based on proved indices not intuition. *BMJ,* **320**(7233), pp. 469-473.

Chung, C.D., Patel, V.P., Moran, M., Lewis, L.A. and Miceli, M.C., (2000). Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction. *Journal of Immunology (Baltimore, MD.: 1950),* **165**(7), pp. 3722-3729.

Chu-yuan, H., Jing, P., Yi-sheng, W., He-ping, P., Hui, Y., Chu-xiong, Z., Guo-jian, L. and Guo-qiang, W., (2013). The impact of chemotherapy-associated

neutrophil/lymphocyte counts on prognosis of adjuvant chemotherapy in colorectal cancer. BMC *Cancer,* **13**(1), pp. 177.

Ciocca, D.R., Gago, F.E., Fanelli, M.A. and Calderwood, S.K., (2006). Co-expression of steroid receptors (Estrogen receptor alpha and/or Progesterone receptors) and HER-2/neu: clinical implications. *The Journal of steroid biochemistry and molecular biology,* **102**(1), pp. 32-40.

Clark, R.A., Huang, S.J., Murphy, G.F., Mollet, I.G., Hijnen, D., Muthukuru, M., Schanbacher, C.F., Edwards, V., Miller, D.M., Kim, J.E., Lambert, J. and Kupper, T.S., (2008). Human squamous cell carcinomas evade the immune response by down-regulation of vascular e-selectin and recruitment of Regulatory T-cells. *The Journal of Experimental Medicine,* **205**(10), pp. 2221-2234.

Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S. and Vignali, D.A., (2007). The inhibitory cytokine IL-35 contributes to Regulatory T-cell function. *Nature,* **450**(7169), pp. 566-569.

Coombes, J.L., Siddiqui, K.R., Arancibia-cárcamo, C.V., Hall, J., Sun, C., Belkaid, Y. and Powrie, F., (2007). A functionally specialized population of mucosal CD103 dcs induces FoxP3 Regulatory T-cells via a TGF-β–and retinoic acid–dependent mechanism. *The Journal of Experimental Medicine,* **204**(8), pp. 1757-1764.

Correale, P., Rotundo, M.S., Del Vecchio, M.T., Remondo, C., Migali, C., Ginanneschi, C., Tsang, K.Y., Licchetta, A., Mannucci, S., Loiacono, L., Tassone, P., Francini, G. and Tagliaferri, P., (2010). Regulatory ($FoxP3^+$) T-cell tumor infiltration is a favorable prognostic factor in advanced colon cancer patients undergoing chemo or chemoimmunotherapy. *Journal of Immunotherapy (Hagerstown, MD.: 1997),* **33**(4), pp. 435-441.

Cosmi, L., Liotta, F., Lazzeri, E., Francalanci, M., Angeli, R., Mazzinghi, B., Santarlasci, V., Manetti, R., Vanini, V., Romagnani, P., Maggi, E., Romagnani, S. and Annunziato, F., (2003). Human $CD8^+CD25^+$ thymocytes share phenotypic and functional features with $CD4^+CD25^+$ Regulatory thymocytes. *Blood,* **102**(12), pp. 4107-4114.

Coughlin, C.M., Salhany, K.E., Gee, M.S., Latemple, D.C., Kotenko, S., Ma, X., Gri, G., Wysocka, M., Kim, J.E. and Liu, L., (1998). Tumor cell responses to IFNY affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. *Immunity,* **9**(1), pp. 25-34.

Croft, C., Carter, L., Swain, S.L. and Dutton, R.W., (1994). Generation of polarized antigen-specific $CD8^+$ effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *The Journal of ExperimentalMmedicine,* **180**(5), pp. 1715-1728.

Cubasch, H., Joffe, M., Hanisch, R., Schuz, J., Neugut, A.I., Karstaedt, A., Broeze, N., Van den berg, E., Mccormack, V. and Jacobson, J.S., (2013). Breast cancer characteristics and HIV among 1,092 women in Soweto, South Africa. *Breast cancer Research and Treatment,* **140**(1), pp. 177-186.

Curiel, T.J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-garcia, J.R., Zhang, L., Burow, M., Zhu, Y., Wei, S., Kryczek, I., Daniel, B., Gordon, A., Myers, L., Lackner, A., Disis, M.L., Knutson, K.L., Chen, L. and Zou, W., (2004). Specific recruitment of Regulatory T-cells in Ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature Medicine,* **10**(9), pp. 942-949.

D'alessio, F.R., Tsushima, K., Aggarwal, N.R., West, E.E., Willett, M.H., Britos, M.F., Pipeling, M.R., Brower, R.G., Tuder, R.M., Mcdyer, J.F. and King, L.S., (2009). $CD4^+CD25^+FoxP3^+$ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. *The Journal of Clinical Investigation,* **119**(10), pp. 2898-2913.

Dannull, J., Su, Z., Rizzieri, D., Yang, B.K., Coleman, D., Yancey, D., Zhang, A., Dahm, P., Chao, N., Gilboa, E. and Vieweg, J., (2005). Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of Regulatory T-cells. *The Journal of Clinical Investigation,* **115**(12), pp. 3623-3633.

De Geus-oei, L., Vriens, D., Van Laarhoven, H.W., Van der graaf, Winette TA and Oyen, W.J., (2009). Monitoring and predicting response to therapy with 18f-FDG pet

in colorectal cancer: a systematic review. *Journal of Nuclear Medicine,* **50**(suppl 1), pp. 43s-54s.

De koning, P.J., Kummer, J.A., De Poot, S.A., Quadir, R., Broekhuizen, R., Mcgettrick, A.F., Higgins, W.J., Devreese, B., Worrall, D.M. and Bovenschen, N., (2011). Intracellular serine Protease Inhibitor Serpinb4 inhibits granzyme induced cell death. *Plos One,* **6**(8), pp. e22645.

Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V.K., Strom, T.B. and Robson, S.C., (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on Regulatory T-cells mediates immune suppression. *The Journal of Experimental Medicine,* **204**(6), pp. 1257-1265.

Decker, T., Fischer, G., Bücke, W., Bücke, P., Stotz, F., Grüneberger, A., Gropp-Meier, M., Wiedemann, G., Pfeiffer, C. and Peschel, C., (2012). Increased number of Regulatory T-cells (Tregs) in the peripheral blood of patients with HER-2/neu-positive early breast cancer. *Journal of Ccancer Research and Clinical Oncology,* **138**(11), pp. 1945-1950.

De-en, H., Xiang-song, L., Jie, H., Bai-ling, L., Xiao-feng, W., Yi-gao, S. and Jia, Y., (1988). The effects of radiotherapy on the immune system of patients with nasopharyngeal carcinoma. *The British Journal of Radiology,* (61), pp. 305-308.

Devi, P.U., (2004). Basics of carcinogenesis. *Health administrator,* **17**(1), pp. 16-24.

Dipaolo, R.J., Brinster, C., Davidson, T.S., Andersson, J., Glass, D. and Shevach, E.M., (2007). Autoantigen- specific TGF-beta- induced FoxP3^{+} Regulatory T-cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T-cells. *Journal of Immunology (Baltimore, MD.: 1950),* **179**(7), pp. 4685.

Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G. and Tamada, K., (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nature Medicine,* **8**(8), pp. 793-800.

Dong, H., Zhu, G., Tamada, K. and Chen, L., (1999). B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nature Medicine,* **5**(12), pp. 1365-1369.

Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. and Sallusto, F., (2009). Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T-cells. *Nature Immunology,* **10**(8), pp. 857-863.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, I.J. and Schreiber, R.D., (2002). Cancer Immunoediting: from immunosurveillance to tumor escape. *Nature Immunology,* **3**(11), pp. 991-998.

Dunn, G.P., Old, L.J. and Schreiber, R.D., (2004). The three es of cancer immunoediting. *Annual review of immunology,* **22**, pp. 329-360.

Ek, T., Mellander, L., Andersson, B. and Abrahamsson, J., (2005). Immune reconstitution after childhood acute lymphoblastic leukemia is most severely affected in the high risk group. *Pediatric Blood and Cancer,* **44**(5), pp. 461-468.

Elston, C. and Ellis, I., (1991). Pathological prognostic factors in breast cancer: the value of histological grade in breast cancer: experience from a large study with long‐term follow‐up. *Histopathology,* **19**(5), pp. 403-410.

Emadi, A., Jones, R.J. and Brodsky, R.A., (2009). Cyclophosphamide and cancer: golden anniversary. *Nature reviews clinical oncology,* **6**(11), pp. 638-647.

Endharti, A., Rifa'i, M., Shi, Z. and Fukuoka, Y., (2005). Nakahara cutting edge: CD8 CD122 Regulatory T-cells produce IL-10 to suppress IFN-gamma production and proliferation of CD8 T-cells. *Journal of Immunology,* **175**(11), pp. 7093-7097.

Esteva, F. and Hortobagyi, G., (2004). Prognostic molecular markers in early breast cancer. *Breast cancer research,* **6**(3), pp. 109-118.

Eyal, A., Kemp, M. and Luvhengo, T., (2007). A 10-year audit of burns at kalafong hospital. *Burns,* **33**(3), pp. 393-395.

Fanning, S., Rybicki, L., Kalaycio, M., Andresen, S., Kuczkowski, E., Pohlman, B., Sobecks, R., Sweetenham, J. and Bolwell, B., (2006). Severe mucositis is associated with reduced survival after autologous stem cell transplantation for lymphoid malignancies. *British Journal of Haematology,* **135**(3), pp. 374-381.

Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C. and Parkin, D.M., (2010). Estimates of worldwide burden of cancer in 2008: Globocan (2008). *International Journal of cancer,* **127**(12), pp. 2893-2917.

Fiore, S., Newell, M., Trabattoni, D., Thorne, C., Gray, L., Savasi, V., Tibaldi, C., Ferrazzi, E. and Clerici, M., (2006). Antiretroviral therapy-associated modulation of Th1 and Th2 immune responses in HIV-infected pregnant women. *Journal of Reproductive Immunology,* **70**(1), pp. 143-150.

Flanigan, R.C., Salmon, S.E., Blumenstein, B.A., Bearman, S.I., Roy, V., Mcgrath, P.C., Caton JR., Munshi, N. and Crawford, E.D., (2001). Nephrectomy followed by interferon alfa-2b compared with interferon alfa-2b alone for metastatic renal-cell cancer. *New England Journal of Medicine,* **345**(23), pp. 1655-1659.

Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y., (2003). FoxP3 programs the development and function of $CD4^{+}CD25^{+}$ Regulatory T-cells. *Nature Immunology,* **4**(4), pp. 330-336.

Gabrilovich, D., (2004). Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nature reviews immunology,* **4**(12), pp. 941-952.

Gago, F.E., Tello, O.M., Diblasi, A.M. and Ciocca, D.R., (1998). Integration of Estrogen and Progesterone receptors with pathological and molecular prognostic factors in breast cancer patients. *TheJournal of Steroid biochemistry and molecular biology,* **67**(5), pp. 431-437.

Gallimore, A. and Godkin, A., (2008). Regulatory T-cells and tumour immunity - observations in mice and men. *Immunology,* **123**(2), pp. 157-163.

Gastman, B.R., Johnson, D.E., Whiteside, T.L. and Rabinowich, H., (1999). Caspase-mediated degradation of T-cell receptor zeta- chain. *Cancer Research,* **59**(7), pp. 1422.

Gastman, B.R., Johnson, D.E., Whiteside, T.L. and Rabinowich, H., (2000). Tumor-induced apoptosis of T-lymphocytes: elucidation of intracellular apoptotic events. *Blood,* **95**(6), pp. 2015-2023.

Gatti, R.A. and Good, R.A., (1971). Occurrence of malignancy in immunodeficiency diseases: a literature review. *Cancer,* **28**(1), pp. 89-98.

Gerosa, F., Baldani-guerra, B., Nisii, C., Marchesini, V., Carra, G. and Trinchieri, G., (2002). Reciprocal activating interaction between Natural Killer cells and dendritic cells. *The Journal of Experimental Medicine,* **195**(3), pp. 327-333.

Gershon, R.K. and Kondo, K., (1970). Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology,* **18**(5), pp. 723-737.

Ghiringhelli, F., Larmonier, N., Schmitt, E., Parcellier, A., Cathelin, D., Garrido, C., Chauffert, B., Solary, E., Bonnotte, B. and Martin, F., (2004). $CD4^{+}CD25^{+}$ Regulatory T-cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *European Journal of Immunology,* **34**(2), pp. 336-344.

Glare, P., (2005). Clinical predictors of survival in advanced cancer. *J Support Oncol,* **3**(5), pp. 331-339.

Gnerlich, J., Jeffe, D.B., Deshpande, A.D., Beers, C., Zander, C. and Margenthaler, J.A., (2007). Surgical removal of the primary tumor increases overall survival in patients with metastatic breast cancer: analysis of the 1988–2003 seer data. A*annals of surgical oncology,* **14**(8), pp. 2187-2194.

Godfrey, D.I. and Berzins, S.P., (2007). Control points in NKT-cell development. *Nature Reviews Immunology,* **7**(7), pp. 505-518.

Goldfarb, Y., Sorski, L., Benish, M., Levi, B., Melamed, R. and Ben-eliyahu, S., (2011). Improving postoperative immune status and resistance to cancer metastasis: a combined perioperative approach of immunostimulation and prevention of excessive surgical stress responses. *Annals of surgery,* **253**(4), pp. 798-810.

Gondek, D.C., Lu, L.F., Quezada,S.A., Sakaguchi, S. and Noelle, R.J., (2005). Cutting edge: contact-mediated suppression by $CD4^+CD25^+$ Regulatory cells involves a granzyme b-dependent, perforin-independent mechanism. *Journal of Immunology (Baltimore, MD.: 1950),* **174**(4), pp. 1783-1786.

González, H.D. and Figueras, J., (2007). Effect of surgical resection of metastatic disease on immune tolerance to cancer. how a systemic disease could be controlled by a local therapy. *Clinical and translational oncology,* **9**(9), pp. 571-577.

Goto, S., Sato, M., Kaneko, R., Itoh, M., Sato, S. and Takeuchi, S., (1999). Analysis of Th1 and Th2 cytokine production by peripheral blood mononuclear cells as a parameter of immunological dysfunction in advanced cancer patients. *Cancer Immunology, Immunotherapy,* **48**(8), pp. 435-442.

Grazia Roncarolo, M., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K. and Levings, M.K., (2006). Interleukin 10 - secreting type 1 Regulatory T-cells in rodents and humans. *Immunological reviews,* **212**(1), pp. 28-50.

Griffiths, C.T. and Fuller, A.F., (1978). Intensive surgical and chemotherapeutic management of advanced Ovarian cancer. *The surgical clinics of North America,* **58**(1), pp. 131-142.

Groh, V., Wu, J., Yee, C. and Spies, T., (2002). Tumour-derived soluble mic ligands impair expression of NKG2D and t-cell activation. *Nature,* **419**(6908), pp. 734-738.

Groux, H., O'garra, A., Bigler, M., Rouleau, M., Antonenko, S., De vries, J.E. and Roncarolo, M.G., (1997). A CD4 and plus; T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature,* **389**(6652), pp. 737-742.

Gupta, S., Joshi, K., Wig, J.D. and Arora, S.K., (2007). Intratumoral FoxP3 expression in infiltrating breast carcinoma: its association with clinicopathologic parameters and angiogenesis. *Acta oncologica,* **46**(6), pp. 792-797.

Gusterson, B.A., Gelber, R.D., Goldhirsch, A., Price, K.N., Save-soderborgh, J., Anbazhagan, R., Styles, J., Rudenstam, C.M., Golouh, R. and Reed, R., (1992). Prognostic importance of c-erbb-2 expression in breast cancer. International (ludwig) breast cancer study group. *Journal of clinical oncology : official journal of the american society of clinical oncology,* **10**(7), pp. 1049-1056.

Haas, M., Dimmler, A., Hohenberger, W., Grabenbauer, G.G., Niedobitek, G. and Distel, L.V., (2009). Stromal Regulatory T-cells are associated with a favourable prognosis in gastric cancer of the cardia. BMC *Gastroenterology,* **9**(1), pp. 65.

Habicht, A., Dada, s., Jurewicz, M., Fife, B.T., Yagita, H., Azuma, M., Sayegh, M.H. and Guleria, I., (2007). A link between PDL1 and T regulatory cells in fetomaternal tolerance. *Journal of immunology (Baltimore, MD.: 1950),* **179**(8), pp. 5211-5219.

Haffejee, A. and Angorn, I., (1979). Nutritional status and the nonspecific cellular and humoral immune response in Esophageal carcinoma. *Annals of surgery,* **189**(4), pp. 475.

Hamada, H., De la luz garcia-hernandez, Maria, Reome, J.B., Misra, S.K., Strutt, T.M., Mckinstry, K.K., Cooper, A.M., Swain, S.L. and Dutton, R.W., (2009). TC17, a unique subset of CD8 T-cells that can protect against lethal influenza challenge. *The journal of Immunology,* **182**(6), pp. 3469-3481.

Hanahan, D. and Folkman, J., (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell,* **86**(3), pp. 353-364.

Harris, S.G., Padilla, J., Koumas, L., Ray, D. and Phipps, R.P., (2002). Prostaglandins as modulators of immunity. *Trends in immunology,* **23**(3), pp. 144-150.

Hayes, D., (2010). Measurement of prognostic factors in breast cancer. in: Gralow ,Julie R. Lerner ,R., ed, *uptodate.* Waltham MA, .

Heller, K.N., Gurer, C. and Münz, C., (2006). Virus-specific CD4 T-cells: ready for direct attack. *The journal of experimental medicine,* **203**(4), pp. 805-808.

Hensler, T, Hecker, H., Heeg, K., Heidecke, C.D., Bartels, H., Barthlen, W., Wagner, H., Siewert, J.R. and Holzmann, B., (1997). Distinct mechanisms of immunosuppression as a consequence of major surgery. *Infection and immunity,* **65**(6), pp. 2283-2291.

Hernandez‐Caselles, T., Rubio, G., Campanero, M.R., Del Pozo, M.A., Muro, M., Sanchez‐Madrid, F. and Aparicio, P., (1993). ICAM‐3, The third LFA‐1 counterreceptor, is a co‐stimulatory molecule for both resting and activated T-lymphocytes. *European Journal of Immunology,* **23**(11), pp. 2799-2806.

Hiraoka, N., Onozato, K., Kosuge, T. and Hirohashi, S., (2006). Prevalence of FoxP3+ Regulatory T-cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clinical cancer research : an official Journal of the American association for cancer research,* **12**(18), pp. 5423-5434.

Hodi, F.S., (2007). Cytotoxic T-Lymphocyte-Associated Antigen-4. *Clinical cancer research : an official journal of the American association for cancer research,* **13**(18 pt 1), pp. 5238-5242.

Hodi, F.S., Butler, M, Oble, D.A., Seiden, M.V., Haluska, F.G., Kruse, A., Macrae, S., Nelson, M., Canning, C., Lowy, I., Korman, A., Lautz, D., Russell, S., Jaklitsch, M.T., Ramaiya, N., Chen, T.C., Neuberg, D., Allison, J.P., Mihm, M.C. and Dranoff, G., (2008). Immunologic and clinical effects of antibody blockade of Cytotoxic T-Lymphocyte-Associated Antigen 4 in previously vaccinated cancer patients. *Proceedings of the national academy of sciences of the United States of America,* **105**(8), pp. 3005-3010.

Hodi, F.S., Mihm, M.C., Soiffer, R.J., Haluska, F.G., Butler, M., Seiden, M.V., Davis, T., Henry-spires, R., Macrae, S., Willman, A., Padera, R., Jaklitsch, M.T., Shankar, S., Chen, T.C., Korman, A., Allison, J.P. and Dranoff, G., (2003). Biologic activity of Cytotoxic T-lymphocyte-associated Antigen 4 antibody blockade in previously

vaccinated Metastatic Melanoma and Ovarian carcinoma patients. *Proceedings of the national academy of sciences of the United States of America,* **100**(8), pp. 4712-4717.

Hori, S., Nomura, T. and Sakaguchi, S., `(2003). Control of Regulatory T-cell development by the transcription factor FoxP3. *Science,* **299**(5609), pp. 1057-1061.

Horwitz, D.A., Zheng, S.G. and Gray, J.D., (2008). Natural and TGF-β–induced FoxP3+ $CD4^{+}CD25^{+}$ regulatory T-cells are not mirror images of each other. *Trends in Immunology,* **29**(9), pp. 429-435.

Hoshida, Y., Tsukuma, H., Yasunaga, Y., Xu, N., Fujita, M.Q., Satoh, T., Ichikawa, Y., Kurihara, K., Imanishi, M. and Matsuno, T., (1997). Cancer risk after renal transplantation in Japan. *International journal of cancer,* **71**(4), pp. 517-520.

Huang, M., Stolina, M., Sharma, S., Mao, J.T., Zhu, L., Miller, P.W., Wollman, J., Herschman, H. and Dubinett, S.M., (1998). Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. *Cancer Research,* **58**(6), pp. 1208-1216.

Hurria, A. and Come, S., (2012). Follow-up for breast cancer survivors: Recommendations for surveillance after therapy. in: D Hayes and Dizon, DS, Dudu, R., eds, *uptodate.* waltham MA.

IARC, (2013), Globocan (2012): Estimated cancer incidence, mortality and prevalence worldwide .[Online] Available from: http://globocan.iarc.fr/pages/fact_sheets_cancer.aspx (Accessed: 21/12/2013).

Ishikawa, M., Nishioka, M., Hanaki, N., Miyauchi, T, Kashiwagi, Y., Ioki, H., Kagawa, A. and Nakamura, Y., (2009). Perioperative immune responses in cancer patients undergoing digestive surgeries. *World J Surg Oncol,* **7**(7).

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F. and Sakaguchi, S., (1999). Thymus and autoimmunity: production of $CD25^{+}CD4^{+}$ naturally anergic and suppressive T-cells as a key function of the thymus in

maintaining immunologic self-tolerance. *Journal of Immunology (Baltimore, MD: 1950),* **162**(9), pp. 5317-5326.

Jaberipour, M., Habibagahi, M., Hosseini, A., Habibabad, S.R., Talei, A. and Ghaderi, A., (2010). Increased CTLA-4 and FoxP3 transcripts in peripheral blood mononuclear cells of patients with breast cancer. *Pathology oncology research,* **16**(4), pp. 547-551.

Jarvis, L.B., Matyszak, M.K., Duggleby, R.C., Goodall, J.C., Hall, F.C. and Gaston, J., (2005). Autoreactive human peripheral blood $CD8^+$ T-cells with a Regulatory phenotype and function. *European Journal of Immunology,* **35**(10), pp. 2896-2908.

Jensen, P., Hansen, S., Møller, B., Leivestad, T., Pfeffer, P., Geiran, O., Fauchald, P. and Simonsen, S., (1999). Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens. *Journal of the American academy of dermatology,* **40**(2), pp. 177-186.

Joosten, S.A., Van Meijgaarden, K.E., Savage, N.D., De boer, T., Triebel, F., Van Der Wal, A., De Heer, E., Klein, M.R., Geluk, A. and Ottenhoff, T.H., (2007). Identification of a human $CD8^+$ regulatory T-cell subset that mediates suppression through the chemokine CC chemokine ligand 4. *Proceedings of the National academy of sciences of the United States of America,* **104**(19), pp. 8029-8034.

Jung, E., Moon, H., Cho, B.I., Jeong, C., Joo, Y., Lee, Y., Hong, S., Choi, S., Ha, W. and Kim, J.W., (2007). Galectin‐1 expression in cancer‐associated stromal cells correlates tumor invasiveness and tumor progression in breast cancer. *International Journal of cancer,* **120**(11), pp. 2331-2338.

Kachikwu, E.L., Iwamoto, K.S., Liao, Y., Demarco, j.j., Agazaryan, N., Economou, J.S., Mcbride, W.H. and Schaue, D., (2011). Radiation enhances Regulatory T-cell representation. *International Journal of radiation Oncology* Biology* Physics,* **81**(4), pp. 1128-1135.

Kang, D.H., Weaver, M.T., Park, N.J., Smith, B., Mcardle, T. and Carpenter, J., (2009). Significant impairment in immune recovery after cancer treatment. *Nursing research,* **58**(2), pp. 105-114.

Kaplan, D.H., Shankaran, V., Dighe, A.S., Stockert, E., Aguet, M., Old, L.J. and Schreiber, R.D., (1998). Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. *Proceedings of the national academy of sciences,* **95**(13), pp. 7556-7561.

Kaplan, M.H., (2013). Th9 cells: differentiation and disease. *Immunological reviews,* **252**(1), pp. 104-115.

Karube, K., Ohshima, K., Tsuchiya, T., Yamaguchi, T., Kawano, R., Auzumiya, J., Utsunomiya, A., Harada, M. and Kikuchi, M., (2004). Expression of FoxP3, a key molecule in $CD4^+CD25^+$ Regulatory T-cells, in adult T-cell leukaemia/lymphoma cells. *British Journal of Haematology,* **126**(1), pp. 81-84.

Khattri, R., Cox, T., Yasayko, S. and Ramsdell, F., (2003). In essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells. *Nature immunology,* **4**(4), pp. 337-342.

Khazaie, K. and Von Boehmer, H, (2006). The impact of $CD4^+CD25^+$ Treg on tumor specific $CD8^+$ T-cell cytotoxicity and cancer. *Seminars in cancer biology,* **16**(2), pp. 124-136.

Kim, R, Emi, M. and Tanabe, K., (2007). Cancer immunoediting from immune surveillance to immune escape. *Immunology,* **121**(1), pp. 1-14.

Kingham, T.P., Alatise, O.I., Vanderpuye, V., Casper, C., Abantanga, F.A., Kamara, T.B., Olopade, O.I., Habeebu, M., Abdulkareem, F.B. and Denny, I., (2013). Treatment of cancer in sub-saharan africa. *The Lancet Oncology,* **14**(4), pp. e158-e167.

Kiniwa, Y., Miyahara, Y., Wang, H.Y., Peng, W., Peng, G., Wheeler, T.M., Thompson, T.C., Old, L.J. and Wang, R.F., (2007). $CD8^+FoxP3^+$ regulatory T-cells mediate immunosuppression in prostate cancer. *Clinical cancer research : an official Journal of the american association for cancer research,* **13**(23), pp. 6947-6958.

Klein, G. and Klein, E., (1977). Immune surveillance against virus-induced tumors and nonrejectability of spontaneous tumors: contrasting consequences of host versus

tumor evolution. *Proceedings of the National Academy of Sciences,* **74**(5), pp. 2121-2125.

Ko, K., Yamazaki, S., Nakamura, K., Nishioka, T., Hirota, K., Yamaguchi, T., Shimizu, J., Nomura, T., Chiba, T. and Sakaguchi, S., (2005). Treatment of advanced tumors with agonistic anti-GITR mab and its effects on tumor-infiltrating $FoxP3^{+}CD25^{+}CD4^{+}$ Regulatory T-cells. *The Journal of Experimental Medicine,* **202**(7), pp. 885-891.

Konecny, G., Pauletti, G., Pegram, M., Untch, M., Dandekar, S., Aguilar, Z., Wilson, C., Rong, H.M., Bauerfeind, I., Felber, M., Wang, H.J., Beryt, M., Seshadri, R., Hepp, H. and Slamon, D.J., (2003). Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. *Journal of the National Cancer Institute,* **95**(2), pp. 142-153.

Kono, K., Kawaida, H., Takahashi, A., Sugai, H., Mimura, K., Miyagawa, N., Omata, H. and Fujii, H., (2006). $CD4^{+}CD25^{+}$high Regulatory T-cells increase with tumor stage in patients with gastric and esophageal cancers. *Cancer Immunology, Immunotherapy,* **55**(9), pp. 1064-1071.

Koretzky, G.A., (2010). Multiple roles of $CD4^{+}$ and $CD8^{+}$ in T-cell activation. *The Journal of Immunology,* **185**(5), pp. 2643-2644.

Kostler, W.J., Schwab, B., Singer, C.F., Neumann, R., Rucklinger, E., Brodowicz, T., Tomek, S., Niedermayr, M., Hejna, M., Steger, G.G., Krainer, M., Wiltschke, C. and Zielinski, C.C., (2004). Monitoring of serum HER-2/neu predicts response and progression-free survival to Trastuzumab-based treatment in patients with metastatic breast cancer. *Clinical Ccancer Research : an official journal of the American association for cancer research,* **10**(5), pp. 1618-1624.

Kostler, W.J., Steger, G.G., Soleiman, A., Schwab, B., Singer, C.F., Tomek, S., Brodowicz, T., Krainer, M., Wiltschke, C., Horvat, R., Jakesz, R. and Zielinski, C.C., (2004). Monitoring of serum HER-2/neu predicts histopathological response to neoadjuvant Trastuzumab-based therapy for breast cancer. *Anticancer research,* **24**(2c), pp. 1127-1130.

Kryczek, I., Wei, S., Zou, L., Zhu, G., Mottram, P., Xu, H., Chen, L. and Zou, W., (2006). Cutting edge: induction of b7-h4 on APCs through IL-10: novel suppressive mode for Regulatory T-cells. *Journal of Immunology (Baltimore, MD.: 1950),* **177**(1), pp. 40-44.

Kumar, A., Commane, M., Flickinger, T.W., Horvath, C.M. and Stark, G.R., (1997). Defective TNF-a-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. *Science,* **278**(5343), pp. 1630-1632.

Kyzas, P.A., Loizou, K.T. and Ioannidis, J.P., (2005). Selective reporting biases in cancer prognostic factor studies. *Journal of the National Cancer Institute,* **97**(14), pp. 1043-1055.

Ladoire, S., Arnould, L., Apetoh, L., Coudert, B., Martin, F., Chauffert, B., Fumoleau, P. and Ghiringhelli, F., (2008). Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating FoxP3^{+} Regulatory T-cells. *Clinical cancer research,* **14**(8), pp. 2413-2420.

Lamont EB, C.N., (2012). Survival estimates in advanced cancer. In: S.D. Morrison RS, ED, *Uptodate.* Waltham, MA: Uptodate.

Lamont, E.B. and Christakis, N.A., (2001). Prognostic disclosure to patients with cancer near the end of life. *Annals of Internal Medicine,* **134**(12), pp. 1096-1105.

Lawrie, D., Coetzee, L.M., Becker, P., Mahlangu, J., Stevens, W. and Glencross, D.K., (2009). Local reference ranges for full blood count and CD4^{+} lymphocyte count testing. *South African Medical Journal,* **99**(4).

Lechner, M.G., Liebertz, D.J. and Epstein, A.L., (2010). Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *Journal of Immunology (Baltimore, MD.: 1950),* **185**(4), pp. 2273-2284.

Lee, D.K., Hakim, F.T. and Gress, R.E., (2010). The thymus and the immune system: layered levels of control. *Journal of Thoracic oncology: official publication of the International association for the study of lung cancer,* **5**(10 suppl 4), pp. s273-6.

Lehrnbecher, T., Foster, C., Vázquez, N., Mackall, C.L. and Chanock, S.J., (1997). Therapy-induced alterations in host defense in children receiving therapy for cancer. *Journal of Pediatric Hematology/Oncology,* **19**(5), pp. 399-417.

Lehrnbecher, T., Koehl, U., Wittekindt, B., Bochennek, K., Tramsen, L., Klingebiel, T. and Chanock, S.J., (2008). Changes in host defence induced by malignancies and antineoplastic treatment: implication for immunotherapeutic strategies. *Lancet Oncology,* **9**(3), pp. 269-278.

Lennard, T., Shenton, B., Borzotta, A., Donnelly, P., White, M., Gerrie, L.M., Proud, G. and Taylor, R., (1985). The influence of surgical operations on components of the human immune system. *British Journal of Surgery,* **72**(10), pp. 771-776.

Leung, A.M., VU, H.N., Nguyen, K., Thacker, L.R. and Bear, H.D., (2010). Effects of surgical excision on survival of patients with Stage IV breast cancer. *Journal of Surgical Research,* **161**(1), pp. 83-88.

Li, C., Uribe, D. and Daling, J., (2005). Clinical characteristics of different histologic types of breast cancer. *British Journal of Cancer,* **93**(9), pp. 1046-1052.

Li, M.O. and Flavell, R.A., (2008). TGF-β: a master of all T-cell trades. *Cell,* **134**(3), pp. 392-404.

Liakos. D, Dower, D., Florizoone, M. and Bizos, D., (2010). Is oesophageal stenting for cancer the answer? a report from a secondary hospital in the developing world. *SAJA,* **2**(48), pp. 43-49.

Lilja, H., Ulmert, D. and Vickers, A.J., (2008). Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. *Nature Reviews Cancer,* **8**(4), pp. 268-278.

Lim, H.W., Hillsamer, P., Banham, A.H. and Kim, C.H., (2005). Cutting edge: direct suppression of B-cells by $CD4^+CD25^+$ Regulatory T-cells. *Journal of Immunology (Baltimore, MD.: 1950),* **175**(7), pp. 4180-4183.

Linsley, P.S., Greene, J.L., Tan, P., Bradshaw, J., Ledbetter, J.A., Anasetti, C. and Damle, N.K., (1992). Coexpression and functional cooperation of CTLA-4 and CD28 on activated T-lymphocytes. *The Journal of Experimental Medicine,* **176**(6), pp. 1595-1604.

Lissoni, P., Brivio, F., Fumagalli, L., Messina, G., Meregalli, S., Porro, G., Rovelli, F., Vigore, L., Tisi, E. and D'amico, G., (2009). Effects of the conventional antitumor therapies surgery, chemotherapy, radiotherapy and immunotherapy on Regulatory T-lymphocytes in cancer patients. *Anticancer research,* **29**(5), pp. 1847-1852.

Liu, L., Wu, G., Yao, J.X., Ding, Q. and Huang, S.A., (2008). $CD4^+CD25^+$high Regulatory cells in peripheral blood of cancer patients. *Neuroendocrinology letters,* **29**(2), pp. 240-245.

Liu, W., Putnam, A.L., Xu-yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., Fazekas De ST Groth, B., Dlayberger, C., Soper, D.M., Ziegler, S.F. and Bluestone, J.A., (2006). $CD127^+$ expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ Treg cells. *Journal of Experimental Medicine,* **203**(7), pp. 1701-1711.

Liyanage, U.K., Moore, T.T., Joo, H.G., Tanaka, Y., Herrmann, V., Doherty, G., Drebin, J.A., Strasberg, S.M., Eberlein, T.J., Goedegebuure, P.S. and Linehan, D.C., (2002). Prevalence of Regulatory T-cells is increased in peripheral blood and tumor microenvironment of patients with Pancreas or Breast adenocarcinoma. *Journal of Immunology,* **169**(5), pp. 2756-2761.

Lockhart, D.C., Chan, A.K., Mak, S., Joo, H., Daust, H.A., Carritte, A., Douville, C.C., Goedegebuure, P.S. and Eberlein, T.J., (2001). Loss of T-cell receptor-CD3ζ and T-cell function in tumor-infiltrating lymphocytes but not in tumor-associated lymphocytes in ovarian carcinoma. *Surgery,* **129**(6), pp. 749-756.

Loeb, L.A., Loeb, K.R. and Anderson, J.P., (2003). Multiple mutations and cancer. *Proceedings of the National academy of Sciences,* **100**(3), pp. 776-781.

Lu, L. and Cantor, H., (2008). Generation and regulation of $CD8^+$ Regulatory T-cells. *Cell Mol Immunol,* **5**(6), pp. 401-406.

Lundgren, A., Stromberg, E., Sjoling, A., Lindholm, C., Enarsson, K., Edebo, A., Johnsson, E., Suri-payer, E., Larsson, P., Rudin, A., Svennerholm, A.M. and Lundin, B.S., (2005). Mucosal FoxP3-expressing $CD4^+CD25^+$high Regulatory T-cells in Helicobacter pylori-infected patients. *Infection and Immunity,* **73**(1), pp. 523-531.

Luster, A.D. and Leder, P., (1993). IP-10, A-cxc-chemokine, elicits a potent thymus-dependent antitumor response in vivo. *The Journal of Experimental Medicine,* **178**(3), pp. 1057-1065.

Lutsiak, M.E., Semnani, R.T., De Pascalis, R., Kashmiri, S.V., Schlom, J. and Sabzevari, H., (2005). Inhibition of $CD4^+25^+$ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood,* **105**(7), pp. 2862-2868.

Mafune, K. and Tanaka, Y., (2000). Influence of multimodality therapy on the cellular immunity of patients with esophageal cancer. *Annals of Surgical Oncology,* **7**(8), pp. 609-616.

Manoukian, G. and Hagemeister, F., (2009). Denileukin Diftitox: a novel immunotoxin. *Expert Opinion on Biological Therapy,* **9**(11), pp. 1445-1451.

Marincola, F.M., Jaffee, E.M., Hicklin, D.J. and Ferrone, S., (2000). Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Advances in Immunology,* **74**, pp. 181-273.

Martorelli, D., Muraro, E., Merlo, A., Turrini, R., Rosato, A. and Dolcetti, R., (2010). Role of $CD4^+$ Cytotoxic T-lymphocytes in the control of viral diseases and cancer. *International Reviews of Immunology,* **29**(4), pp. 371-402.

Mellor, A.L. and Munn, D.H., (2004). IDO expression by dendritic cells: tolerance and Tryptophan catabolism. *Nature reviews immunology,* **4**(10), pp. 762-774.

Mendonça, M.A.O., Cunha, F.Q., Murta, E.F.C. and Tavares-Murta, B.M., (2006). Failure of neutrophil chemotactic function in breast cancer patients treated with chemotherapy. *Cancer Chemotherapy and Pharmacology,* **57**(5), pp. 663-670.

Merlo, A., Casalini, P., Carcangiu, M.L., Malventano, C., Triulzi, T., Menard, S., Tagliabue, E. and Balsari, A., (2009). FoxP3 expression and overall survival in breast cancer. *Journal of Clinical Oncology,* **27**(11), pp. 1746-1752.

Mitri, Z., Constantine, T. and O'regan, R., (2012). The HER2 receptor in breast cancer: pathophysiology, clinical use, and new advances in therapy. *Chemotherapy Research and Practice, pp* 1-5.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., Mathian, A., Nakahata, T., Yamaguchi, T., Nomura, T., Ono, M., Amoura, Z., Gorochov, G. and Sakaguchi, S., (2009). Functional delineation and differentiation dynamics of human $CD4^+$ T-cells expressing the FoxP3 transcription factor. *Immunity,* **30**(6), pp. 899-911.

Morgan, G., (2003). Chemotherapy and the cell cycle. *Cancer Nursing Practice,* **2**(1), pp. 27-30.

Morhason-bello, I.O., Odedina, F., Rebbeck, T.R., Harford, J., Dangou, J., Denny, L. and Adewole, I.F., (2013). Challenges and opportunities in cancer control in Africa: a perspective from the African organisation for research and training in cancer. *The Lancet oncology,* **14**(4), pp. e142-e151.

Morse, M.A., Hall, J.R. and Plate, J.M., (2009). Countering tumor-induced immunosuppression during immunotherapy for Pancreatic cancer.

Mosmann, T.R. and Sad, S., (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today,* **17**(3), pp. 138-146.

Mourad-zeidan, A.A., Melnikova, V.O., Wang, H., Raz, A. and Bar-eli, M., (2008). Expression profiling of Galectin-3-depleted Melanoma cells reveals its major role in melanoma cell plasticity and vasculogenic mimicry. *The American Journal of pathology,* **173**(6), pp. 1839-1852.

Mumberg, D., Monach, P.A., Wanderling, S., Philip, M., Toledano, A.Y., Schreiber, R.D. and Schreiber, H., (1999). CD^+ T-cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-γ. *Proceedings of the National Academy of Sciences,* **96**(15), pp. 8633-8638.

Munn, D.H. and Mellor, A.L., (2007). Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *The Journal of Clinical Investigation,* **117**(5), pp. 1147-1154.

Murphy, K., (2012). Principles of Innate and Adaptive Immunity. *Janeway's Immunobiology 8th ed*. New York, USA: Garland Science, pp. 3-36.

Murphy, K., (2012b). The development of T-lymphocytes in the thymus. *Janeway's Immunobiology 8th ed.* Yew York, USA: Garland Science, pp. 290-315.

Murphy, K., (2012c). Priming of naive T-cells by pathogen-activated dendritic cells. *Janeway's Immunobiology 8th ed.* New York, USA: Garland Science, pp. 353-365.

Murphy, K., (2012d). Manipulation of the immune response. *Janeway's Immunobiology 8th ed.* New York, USA: Garland Science, pp. 669-711.

Nagai, H., Horikawa, T., Hara, I., Fukunaga, A., Oniki, S., Oka, M., Nishigori, C. and Ichihashi, M., (2004). In vivo elimination of $CD25^+$ Regulatory T-cells leads to tumor rejection of B16F10 melanoma, when combined with interleukin ‐ 12 gene transfer. *Experimental Dermatology,* **13**(10), pp. 613-620.

Nagaraj, S., Gupta, K., Pisarev, V., Kinarsky, L., Sherman, S., Kang, L., Herber, D.L., Schneck, J. and Gabrilovich, D.I., (2007). Altered recognition of antigen is a mechanism of $CD8^+$ T-cell tolerance in cancer. *Nature Medicine,* **13**(7), pp. 828-835.

Naik, J.D., Sathiyaseelan, S.R. and Vasudev, N.S., (2010). Febrile neutropenia. *BMJ (Clinical Research ed.),* **341**, pp. c6981.

Nakamura, R., Sakakibara, M., Nagashima, T., Sangai, T., Arai, M., Fujimori, T., Takano, S., Shida, T., Nakatani, Y. and Miyazaki, M., (2009). Accumulation of Regulatory T-cells in sentinel lymph nodes is a prognostic predictor in patients with node-negative breast cancer. *European Journal of Cancer,* **45**(12), pp. 2123-2131.

Nakamura, T., Shima, T., Saeki, A., Hidaka, T., Nakashima, A., Takikawa, O. and Saito, S., (2007). Expression of Indoleamine 2, 3 - dioxygenase and the recruitment of $FoxP3^+$ expressing Regulatory T-cells in the development and progression of uterine cervical cancer. *Cancer Science,* **98**(6), pp. 874-881.

Nakamura, R., Sakakibara, M., Nagashima, T., Sangai, T., Arai, M., Fujimori, T., Takano, S., Shida, T., Nakatani, Y. and Miyazaki, M., (2009). Accumulation of Regulatory T-cells in sentinel lymph nodes is a prognostic predictor in patients with node-negative breast cancer. *European Journal of Cancer,* **45**(12), pp. 2123-2131.

National Health Laboratory Services, (2014), National cancer registry 2006. [Online] Available from: http://www.nioh.ac.za/?page=2006_summary_informationandid=183 (Accessed on: 23/03/2014).

Nguyen, D.X. and Massagué, J., (2007). Genetic determinants of cancer metastasis. *Nature Reviews Genetics,* **8**(5), pp. 341-352.

Nicholson, J.K., Hubbard, M. and Jones, B.M., (1996). Use of CD45 fluorescence and side - scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry. *Cytometry,* **26**(1), pp. 16-21.

Nowicka, A., Rogala, E., Bednarek, W., Barczyński, B., Wertel, I., Piekarczyk, W. and Kotarski, J., (2012). Lymphocyte activation markers in patients with ovarian cancer. *Ginekologia Polska,* **83**(10), pp. 737-743.

O'day, S.J., Maio, M., Chiarion-Sileni, V., Gajewski, T.F., Pehamberger, H., Bondarenko, I.N., Queirolo, P., Lundgren, L., Mikhailov, S., Roman, L.,

Verschraegen, C., Humphrey, R., Ibrahim, R., De pril, V., Hoos, A. and Wolchok, J.D., (2010). Efficacy and safety of Ipilimumab monotherapy in patients with pretreated advanced Melanoma: a multicenter single-arm phase II study. *Annals of oncology : Official Journal of the European society for medical oncology / esmo,* **21**(8), pp. 1712-1717.

Oderup, C., Cederbom, L., Makowska, A., Cilio, C.M. and Ivars, F., (2006). Cytotoxic T-lymphocyte antigen ‐ 4 ‐ dependent down ‐ modulation of costimulatory molecules on dendritic cells in $CD4^{+}CD25^{+}$ Regulatory T ‐ cell ‐ mediated suppression. *Immunology,* **118**(2), pp. 240-249.

Ogawa, K., Hirai, M., Katsube, T., Murayama, M., Hamaguchi, K., Shimakawa, T., Naritake, Y., Hosokawa, T. and Kajiwara, T., (2000). Suppression of cellular immunity by surgical stress. *Surgery,* **127**(3), pp. 329-336.

Ohara, M., Yamaguchi, Y., Matsuura, K., Murakami, S., Arihiro, K. and Okada, M., (2009). Possible involvement of Regulatory T-cells in tumor onset and progression in primary breast cancer. *Cancer Immunology, Immunotherapy,* **58**(3), pp. 441-447.

Old, L.J., Boyse, E.A., Clarke, D.A. and Carswell, E.A., (1962). Antigenic properties of chemically induced tumors*. *Annals of the New York academy of sciences,* **101**(1), pp. 80-106.

Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T. and Nakayama, E., (1999). Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Research,* **59**(13), pp. 3128-3133.

Orentas, R.J., Kohler, M.E. and Johnson, B.D., (2006). Suppression of anti-cancer immunity by Regulatory T-cells: back to the future,. *Seminars in cancer biology* 2006, Elsevier, pp. 137-149.

Ormandy, L.A., Hillemann, T., Wedemeyer, H., Manns, M.P., Greten, T.F. and Korangy, F., (2005). Increased populations of Regulatory T-cells in peripheral blood of patients with Hepatocellular carcinoma. *Cancer Research,* **65**(6), pp. 2457-2464.

Paik, S., Bryant, J., Park, C., Fisher, B., Tan-chiu, E., Hyams, D., Fisher, E.R., Lippman, M.E., Wickerham, D.L. and Wolmark, N., (1998). Erbb-2 and response to Doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative Breast cancer. *Journal of the National Cancer Institute,* **90**(18), pp. 1361-1370.

Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. and Lenardo, M.J., (2007). $CD4^+CD25^+$ $FoxP3^+$ Regulatory T-cells induce cytokine deprivation–mediated apoptosis of effector $CD4^+$ T-cells. *Nature Immunology,* **8**(12), pp. 1353-1362.

Pardoll, D.M. and Topalian, S.L., (1998). The role of $CD4^+$ T-cell responses in antitumor immunity. *Current opinion in Immunology,* **10**(5), pp. 588-594.

Parkin, D.M., Bray, F., Ferlay, J. and Pisani, P., (2001). Estimating the world cancer burden: Globocan 2000. *International Journal of Cancer,* **94**(2), pp. 153-156.

Parry, R.V., Chemnitz, J.M., Frauwirth, K.A., Lanfranco, A.R., Braunstein, I., Kobayashi, S.V., Linsley, P.S., Thompson, C.B. and Riley, J.L., (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Molecular and Cellular Biology,* **25**(21), pp. 9543-9553.

Pasquet, L., Douet, J.Y., Sparwasser, T., Romagnoli, P. and Van Meerwijk, J.P., (2013). Long-term prevention of chronic allograft rejection by Regulatory T-cell immunotherapy involves host FoxP3-expressing T-cells. *Blood,* **121**(21), pp. 4303-4310.

Patel, A.N., Preskitt, J.T., Kuhn, J.A., Hebeler, R.F., Wood, R.E. and Urschel, H.C.,JR, (2003). Surgical management of Esophageal carcinoma. *Proceedings (Baylor University.Medical Center),* **16**(3), pp. 280-284.

Peli, J., Schröter, M., Rudaz, C., Hahne, M., Meyer, C., Reichmann, E. and Tschopp, J., (1999). Oncogenic ras inhibits fas ligand ‐ mediated apoptosis by downregulating the expression of fas. *The Embo Journal,* **18**(7), pp. 1824-1831.

Penn, I., (2000). Post-transplant malignancy. *Drug safety,* **23**(2), pp. 101-113.

Perez, S.A., Karamouzis, M.V., Skarlos, D.V., Ardavanis, A., Sotiriadou, N.N., Iliopoulou, E.G., Salagianni, M.L., Orphanos, G., Baxevanis, C.N., Rigatos, G. and Papamichail, M., (2007). $CD4^+CD25^+$ Regulatory T-cell frequency in HER-2/neu (HER)-positive and HER-negative advanced-stage breast cancer patients. *Clinical Cancer Research,* **13**(9), pp. 2714-2721.

Perillo, N.L., Pace, K.E., Seilhamer, J.J. and Baum, L.G., (1995). Apoptosis of T-cells mediated by Galectin-1: 736-739

Petersen, R.P., Campa, M.J., Sperlazza, J., Conlon, D., Joshi, M., Harpole, D.H. and Patz, E.F., (2006). Tumor infiltrating $FoxP3^+$ Regulatory T - cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer,* **107**(12), pp. 2866-2872.

Pickens, A. and Orringer, M.B., (2003). Geographical distribution and racial disparity in Esophageal cancer. *The annals of thoracic surgery,* **76**(4), pp. s1367-s1369.

Pollock, R.E., Lotzova, E. and Stanford, S.D., (1991). Mechanism of surgical stress impairment of human perioperative natural killer cell cytotoxicity. *Archives of surgery (Chicago, Ill.: 1960),* **126**(3), pp. 338-342.

Qiao, M., Thornton, A.M. and Shevach, E.M., (2007). $CD4^+CD25^+$ regulatory T-cells render naive $CD4^+$ $CD25^-$ T-cells anergic and suppressive. *Immunology,* **120**(4), pp. 447-455.

Qin, Z. and Blankenstein, T., (2000). $CD4^+$ T-cell–mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN-γ receptor expression by nonhematopoietic cells. *Immunity,* **12**(6), pp. 677-686.

Rakha, E.A., EL - Sayed, M.E., Green, A.R., Lee, A.H., Robertson, J.F. and Ellis, I.O., (2007). Prognostic markers in Triple - negative breast cancer. *Cancer,* **109**(1), pp. 25-32.

Ramsdell, F., (2003). FoxP3 and natural Regulatory T-cells: key to a cell lineage? *Immunity,* **19**(2), pp. 165-168.

Rasmussen, L. and Arvin, A., (1982). Chemotherapy-induced immunosuppression. *Environmental Health Perspectives,* **43**, pp. 21-25.

Ray-coquard, I., Cropet, C., Van Glabbeke, M., Sebban, C., LE Cesne, A., Judson, I., Tredan, O., Verweij, J., Biron, P., Labidi, I., Guastalla, J.P., Bachelot, T., Perol, D., Chabaud, S., Hogendoorn, P.C., Cassier, P., Dufresne, A., Blay, J.Y. and European organization for research and treatment of cancer soft tissue and bone Sarcoma,group, (2009). Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. *Cancer Research,* **69**(13), pp. 5383-5391.

Rech, A.J., Mick, R., Kaplan, D.E., Chang, K.M., Domchek, S.M. and Vonderheide, R.H., (2010). Homeostasis of peripheral FoxP3^{+} CD4^{+} Regulatory T-cells in patients with early and late stage breast cancer. *Cancer Immunology, Immunotherapy,* **59**(4), pp. 599-607.

Reddy, M., Eirikis, E., Davis, C., Davis, H.M. and Prabhakar, U., (2004). Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function. *Journal of Immunological methods,* **293**(1), pp. 127-142.

Reichert, T.E., Day, R., Wagner, E.M. and Whiteside, T.L., (1998). Absent or low expression of the Zeta chain in T-cells at the tumor site correlates with poor survival in patients with oral carcinoma. *Cancer research,* **58**(23), pp. 5344-5347.

Riley, R., Sauerbrei, W. and Altman, D., (2009). Prognostic markers in cancer: the evolution of evidence from single studies to meta-analysis, and beyond. *British Journal of cancer,* **100**(8), pp. 1219-1229.

Rodríguez, P.C. and Ochoa, A.C., (2008). Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunological reviews,* **222**(1), pp. 180-191.

Ronchetti, S., Zollo, O., Bruscoli, S., Agostini, M., Bianchini, R., Nocentini, G., Ayroldi, E. and Riccardi, C., (2004). Frontline: GITR, a member of the TNF receptor

superfamily, is costimulatory to mouse T-lymphocyte subpopulations. *European Journal of Immunology,* **34**(3), pp. 613-622.

Rosen, S.A., Buell, J.F., Yoshida, A., Kazsuba, S., Hurst, R., Michelassi, F., Millis, J.M. and Posner, M.C., (2000). Initial presentation with Stage IV colorectal cancer: how aggressive should we be? *Archives of Surgery,* **135**(5), pp. 530-534.

Ruffell, B., Denardo, D.G., Affara, N.I. and Coussens, L.M., (2010). Lymphocytes in cancer development: polarization towards pro-tumor immunity. *Cytokine and growth factor reviews,* **21**(1), pp. 3-10.

Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T. and Nomura, T., (2006). FoxP3 $CD25^{+}CD4^{+}$ natural Regulatory T-cells in dominant self‐tolerance and autoimmune disease. *Immunological reviews,* **212**(1), pp. 8-27.

Sakaguchi, S., Yamaguchi, T., Nomura, T. and Ono, M., (2008). Regulatory T-cells and Immune tolerance. *Cell,* **133**(5), pp. 775-787.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M., (1995). Immunologic self-tolerance maintained by activated T-cells expressing IL-2 receptor alpha-chains (CD25). breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of Immunology (Baltimore,MD.: 1950),* **155**(3), pp. 1151-1164.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M., (1995). Immunologic self-tolerance maintained by activated T-cells expressing IL-2 receptor alpha-chains (CD25). breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of immunology,* **155**(3), pp. 1151-1164.

Salama, P., Phillips, M., Grieu, F., Morris, M., Zeps, N., Joseph, D., Platell, C. and Iacopetta, B., (2009). Tumor-infiltrating $FoxP3^{+}$ T regulatory cells show strong prognostic significance in colorectal cancer. *journal of clinical oncology : official Journal of the American society of clinical oncology,* **27**(2), pp. 186-192.

Sasada, T., Kimura, M., Yoshida, Y., Kanai, M. and Takabayashi, A., (2003). $CD4^+$ $CD25^+$ Regulatory T-cells in patients with gastrointestinal malignancies. *cancer,* **98**(5), pp. 1089-1099.

Schmielau, J., Nalesnik, M.A. and Finn, O.J., (2001). Suppressed T-cell receptor ζ chain expression and cytokine production in Pancreatic cancer patients. *Cancer Research,* **7**(3 supplement), pp. 933s-939s.

Schmielau, J. and Finn, O.J., (2001). activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Research,* **61**(12), pp. 4756-4760.

Schnitt, S. and Guidi, A., (2004). Pathology of Ivasive Breast cancer. in: J.R. Harris, M.E. Lippman, C.K. Osborne and M. Morrow, eds, *Diseases of the breast.* 3rd edn. Philadelphia: Lippincott Williams and Wilkins, pp. 393.

Schumacher, K., Haensch, W., Roefzaad, C. and Schlag, P.M., (2001). Prognostic significance of activated $CD8^+$ T-cell infiltrations within Esophageal carcinomas. *Cancer Research,* **61**(10), pp. 3932-3936.

Seddiki, N., Santner-nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S.I., Nanan, R., Kelleher, A. and Fazekas De St Groth, B., (2006). Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human Regulatory and activated T-cells. *The Journal of experimental medicine,* **203**(7), pp. 1693-1700.

Sellitto, A., Galizia, G., De Fanis, U., Lieto, E., Zamboli, A., Orditura, M., De Vita, F., Giunta, R., Lucivero, G. and Romano, C., (2011). Behavior of circulating $CD4^+$ $CD25^+$ $FoxP3^+$ Regulatory T-cells in colon cancer patients undergoing surgery. *Journal of Clinical Immunology,* **31**(6), pp. 1095-1104.

Seremet, T., Brasseur, F. and Coulie, P.G., (2011). Tumor-specific antigens and Immunologic adjuvants in cancer immunotherapy. *Cancer Journal,* **17**(5), pp. 325-330.

Setoguchi, R., Hori, S., Takahashi, T. and Sakaguchi, S., (2005). Homeostatic maintenance of natural FoxP3$^+$ CD25$^+$ CD4$^+$ Regulatory T-cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *Journal of Experimental Medicine,* **201**(5), pp. 723-735.

Sewell, H.F., Halbert, C.F., Robins, R.A., Galvin, A., Chan, S. and Blamey, R.W., (1993). Chemotherapy-induced differential changes in lymphocyte subsets and Natural-Killer-cell function in patients with advanced Breast cancer. *International Journal of Cancer,* **55**(5), pp. 735-738.

Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J. and Schreiber, R.D., (2001). IFN-γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature,* **410**(6832), pp. 1107-1111.

Sheil, A., (1984). Cancer in organ transplant recipients: part of an induced immune deficiency syndrome. *British Medical Journal (Clinical Research ed.),* **288**(6418), pp. 659.

Shevach, E.M., Davidson, T.S., Huter, E.N., Dipaolo, R.A. and Andersson, J., (2008). Role of TGF-β in the induction of FoxP3 expression and T regulatory cell function. *Journal of Clinical Immunology,* **28**(6), pp. 640-646.

Shevach, E.M., (2002). CD4$^+$CD25$^+$ Suppressor T-cells: more questions than answers. *Nature reviews.Immunology,* **2**(6), pp. 389-400.

Simonetta, F., Chiali, A., Cordier, C., Urrutia, A., Girault, I., Bloquet, S., Tanchot, C. and Bourgeois, C., (2010). Increased CD127$^+$ expression on activated FoxP3$^+$ CD4$^+$ Regulatory T-cells. *European Journal of Immunology,* **40**(9), pp. 2528-2538.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J. and Pegram, M., (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England Journal of Medicine,* **344**(11), pp. 783-792.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and Mcguire, W.L., (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science (New York, n.y.),* **235**(4785), pp. 177-182.

Small, E.J., Tchekmedyian, N.S., Rini, B.I., Fong, L., Lowy, I. and Allison, J.P., (2007). A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer. *Clinical Cancer Research : an official journal of the American association for cancer research,* **13**(6), pp. 1810-1815.

Smuts, I., Louw, R., Du Toit, H., Klopper, B., Mienie, L.J. and Van der westhuizen, Francois H, (2010). An overview of a cohort of South African patients with mitochondrial disorders. *Journal of Inherited Metabolic Disease,* , pp. 1-10.

Smyth, M.J., Cretney, E., Takeda, K., Wiltrout, R.H., Sedger, L.M., Kayagaki, N., Yagita, H. and Okumura, K., (2001). Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) contributes to Interferon γ–dependent Natural Killer Cell protection from tumor metastasis. *The Journal of Experimental Medicine,* **193**(6), pp. 661-670.

Smyth, M.J., Thia, K.Y., Cretney, E., Kelly, J.M., Snook, M.B., Forbes, C.A. and Scalzo, A.A., (1999). Perforin is a major contributor to NK cell control of tumor metastasis. *The Journal of Immunology,* **162**(11), pp. 6658-6662.

Standish, L.J., Sweet, E.S., Novack, J., Wenner, C.A., Bridge, C., Nelson, A., Martzen, M. and Torkelson, C., (2008). Breast cancer and the immune system. *Journal of the Society for Integrative Oncology,* **6**(4), pp. 158-168.

Standish, L.J., Torkelson, C., Hamill, F.A., Yim, D., Hill-force, A., Fitzpatrick, A., Olsen, M., Schildt, S., Sweet, E., Wenner, C.A. and Martzen, M.R., (2008). Immune defects in Breast cancer patients after radiotherapy. *Journal of the Society for Integrative Oncology,* **6**(3), pp. 110-121.

Stanzani, M., Martins, S.L., Saliba, R.M., John, L.S.S., Bryan, S., Couriel, D., Mcmannis, J., Champlin, R.E., Molldrem, J.J. and Komanduri, K.V., (2004). $CD25^{+}$ expression on donor $CD4^{+}$ or $CD8^{+}$ T-cells is associated with an increased risk for

graft-versus-host disease after HLA-identical stem cell transplantation in humans. *Blood,* **103**(3), pp. 1140-1146.

Starska, K., Głowacka, E., Kulig, A., Lewy-trenda, I., Bryś, M. and Lewkowicz, P., (2011). The role of tumor cells in the modification of T-lymphocytes activity-the expression of the early $CD69^+$, $CD71^+$ and the late $CD25^+$, $CD26^+$, HLA/DR activation markers on $CD4^+$ and $CD8^+$ T-cells in squamous cell laryngeal carcinoma. part i. *Folia Histochem Cytobiol,* **49**, pp. 579-592.

Stats SA, (2013), Mid-year population estimates. [Online] Available from: http://beta2.statssa.gov.za/publications/sastatistics/sastatistics2013.pdf (Accessed: 23/03/2014).

Staudt, V., Bothur, E., Klein, M., Lingnau, K., Reuter, S., Grebe, N., Gerlitzki, B., Hoffmann, M., Ulges, A. and Taube, C., (2010). Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity,* **33**(2), pp. 192-202.

Storie, I., (2005), Total cell counting. [Online] Available from: http://www.dako.com/08065_15dec05_guide_to_flow_cytometry_total_cell_counting_chapter17.pdf (Accessed: 17/01/2013).

Strauss, L., Bergmann, C., Gooding, W., Johnson, J.T. and Whiteside, T.L., (2007). The frequency and suppressor function of $CD4^+CD25^+$highFoxP3$^+$ T-cells in the circulation of patients with squamous cell carcinoma of the head and neck. *Clinical cancer research : an official Journal of the American Association for Cancer Research,* **13**(21), pp. 6301-6311.

Street, S.E., Hayakawa, Y., Zhan, Y., Lew, A.M., Macgregor, D., Jamieson, A.M., Diefenbach, A., Yagita, H., Godfrey, D.I. and Smyth, M.J., (2004). Innate immune surveillance of spontaneous B-cell lymphomas by Natural Killer cells and γδ T-cells. *The Journal of Experimental Medicine,* **199**(6), pp. 879-884.

Suchard, M.S., Mayne, E., Green, V.A., Shalekoff, S., Donninger, S.L., Stevens, W.S., Gray, C.M. and Tiemessen, C.T., (2010.) FoxP3 expression is upregulated in

CD4$^+$ T-cells in progressive HIV-1 infection and is a marker of disease severity. *Plos One,* **5**(7), pp. e11762.

Sutmuller, R.P., Van Duivenvoorde, L.M., Van Elsas, A., Schumacher, T.N., Wildenberg, M.E., Allison, J.P., Toes, R.E., Offringa, R. and Melief, C.J., (2001). Synergism of Cytotoxic T-lymphocyte-associated Antigen 4 blockade and depletion of CD25$^+$ Regulatory T-cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T-lymphocyte responses. *The Journal of Experimental Medicine,* **194**(6), pp. 823-832.

Szczepanik, A.M., Siedlar, M., Sierzega, M., Goroszeniuk, D., Bukowska-Strakova, K., Czupryna, A. and Kulig, J., (2011). T-Regulatory lymphocytes in peripheral blood of gastric and colorectal cancer patients. *World Journal of Gastroenterology: WJG,* **17**(3), pp. 343.

Taams, L.S., Palmer, D.B., Akbar, A.N., Robinson, D.S., Brown, Z. and Hawrylowicz, C.M., (2006). Regulatory T-cells in human disease and their potential for therapeutic manipulation. *Immunology,* **118**(1), pp. 1-9.

Takeda, K., Hayakawa, Y., Smyth, M.J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H. and Okumura, K., (2001). Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver Natural Killer cells. *Nature Medicine,* **7**(1), pp. 94-100.

Tannock, I.F., Hill, R.P., Bristow, R.G. and Harrington, L., (1998). *The basic science of oncology.* Mcgraw-hill New York.

Teng, M.W., Ritchie, D.S., Neeson, P. and Smyth, M.J., (2011). Biology and clinical observations of Regulatory T-cells in cancer immunology. *Cancer Immunology and Immunotherapy.* Springer, pp. 61-95.

Terabe, M., Matsui, S., Park, J.M., Mamura, M., Noben-trauth, N., Donaldson, D.D., Chen, W., Wahl, S.M., Ledbetter, S., Pratt, B., Letterio, J.J., Paul, W.E. and Berzofsky, J.A., (2003). Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T-cells block

Cytotoxic T-lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *The Journal of Experimental medicine,* **198**(11), pp. 1741-1752.

Tettey, M., Edwin, F., Aniteye, E., Sereboe, L., Tamatey, M., Ofosu-appiah, E. and Adzamli, I., (2013). The changing epidemiology of Esophageal cancer in Sub-saharan africa–the case of Ghana. *Pan african Medical Journal,* **13**(1),.

Tohyama, N., Tanaka, S., Onda, K., Sugiyama, K. and Hirano, T., (2013). Influence of anticancer agents on cell survival, proliferation, and $CD4^{+}CD25^{+}$FoxP3+ Regulatory T-cell-frequency in human peripheral-blood mononuclear cells activated by T-cell-mitogen. *International Immunopharmacology,* **15**(1), pp. 160-166.

Töpfer, K., Kempe, S., Müller, N., Schmitz, M., Bachmann, M., Cartellieri, M., Schackert, G. and Temme, A., (2011). Tumor evasion from T-cell surveillance. *Biomed Research International,* **2011**.

Traverso, I., Fenoglio, D., Negrini, S., Parodi, A., Battaglia, F., Kalli, F., Conteduca, G., Tardito, S., Traverso, P., Indiveri, F. and Filaci, G., (2012). Cyclophosphamide inhibits the generation and function of $CD8^{+}$ Regulatory T-cells. *Human Immunology,* **73**(3), pp. 207-213.

Trzonkowski, P., Szmit, E., Mysliwska, J., Dobyszuk, A. and Mysliwski, A., (2004). $CD4^{+}CD25^{+}$ T Regulatory cells inhibit cytotoxic activity of T $CD8^{+}$ and NK lymphocytes in the direct T-cell-to-cell interaction. *Clinical Immunology,* **112**(3), pp. 258-267.

Tsutsui, S., Sonoda, K., Sumiyoshi, K., Kitamura, K., Toh, Y., Kitamura, M., Kuwano, H., Sugimachi, K. and Okamura, S., (1996). Prognostic significance of immunological parameters in patients with esophageal cancer. *Hepato-gastroenterology,* **43**(9), pp. 501-509.

Tuve, S., Chen, B.M., Liu, Y., Cheng, T.L., Toure, P., Sow, P.S., Feng, Q., Kiviat, N., Strauss, R., Ni, S., Li, Z.Y., Roffler, S.R. and Lieber, A., (2007). Combination of tumor site-located CTL-associated antigen-4 blockade and systemic Regulatory T-cell

depletion induces tumor-destructive immune responses. *Cancer Research,* **67**(12), pp. 5929-5939.

United Nations Scientific Committee on the effects of atomic radiation, (2000). *Sources and effects of ionizing radiation: sources.* united nations publications.

Vallböhmer, D. and Lenz, H., (2006). Predictive and prognostic molecular markers in outcome of esophageal cancer. *Diseases of the esophagus,* **19**(6), pp. 425-432.

Van den brûle, F., Califice, S., Garnier, F., Fernandez, P.L., Berchuck, A. and Castronovo, V., (2003). Galectin-1 accumulation in the Ovary carcinoma peritumoral stroma is induced by ovary carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and Fibronectin. *Laboratory investigation,* **83**(3), pp. 377-386.

Veronese, S., Gambacorta, M., Gottardi, O., Scanzi, F., Ferrari, M. and Lampertico, P., (1993). Proliferation index as a prognostic marker in breast cancer. *Cancer,* **71**(12), pp. 3926-3931.

Vieira, P.L., Christensen, J.R., Minaee, S., O'Neill, E.J., Barrat, F.J., Boonstra, A., Barthlott, T., Stockinger, B., Wraith, D.C. and O'Garra, A., (2004). IL-10-secreting Regulatory T-cells do not express FoxP3^{+} but have comparable regulatory function to naturally occurring CD4^{+}CD25^{+} Regulatory T-cells. *The Journal of Immunology,* **172**(10), pp. 5986-5993.

Voo, K.S., Wang, Y.H., Santori, F.R., Boggiano, C., Wang, Y.H., Arima, K., Bover, L., Hanabuchi, S., Khalili, J., Marinova, E., Zheng, B., Littman, D.R. and Liu, Y.J., (2009). Identification of IL-17-producing FoxP3^{+} Regulatory T-cells in humans. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(12), pp. 4793-4798.

Vorobiof, D.A., Sitas, F. and Vorobiof, G., (2001). Breast cancer incidence in South Africa. *Journal of Clinical oncology,* **19**(suppl 1), pp. 125s-127s.

Wadler, B.M., Judge, C.M., Prout, M., Allen, J.D. and Geller, A.C., (2011). Improving breast cancer control via the use of community health workers in South Africa: a critical review. *Journal of oncology,* **2011**, pp. 10.1155/2011/150423. epub 2010 sep 26.

Wan, Y.Y. and Flavell, R.A., (2007). Regulatory T-cell functions are subverted and converted owing to attenuated FoxP3 expression. *Nature,* **445**(7129), pp. 766-770.

Wang, W., Wu, T., Chandan, V.S., Lohse, C.M. and Zhang, L., (2011). Ki-67 and Proexc are useful immunohistochemical markers in esophageal squamous intraepithelial neoplasia. *Human pathology,* **42**(10), pp. 1430-1437.

Wang, Z., Yang, B., Liu, H., Hu, Y., Yang, J., Wu, L., Zhou, Z. and Jiao, S., (2012). Regulatory T-cells increase in breast cancer and in stage IV breast cancer. *Cancer Immunology, Immunotherapy,* **61**(6), pp. 911-916.

Watanabe, M.A., Oda, J.M., Amarante, A.K. and Cesar Voltarelli, J., (2010). Regulatory T-cells and breast cancer: Implications for immunopathogenesis. *Cancer and Metastasis reviews,* **29**(4), pp. 569-579.

Webb, S.D., Sherratt, J.A. and Fish, R.G., (2002). Cells behaving badly: a theoretical model for the FAS/FASL system in tumour immunology. *Mathematical biosciences,* **179**(2), pp. 113-129.

Weigelt, B., Peterse, J. and Van't Veer, L., (2005). Breast cancer metastasis: markers and models. *Nature reviews cancer,* **5**(8), pp. 591-602.

Weiner, H.L., (2001). Induction and mechanism of action of transforming growth factor - β - secreting TH3 Regulatory cells. *Immunological reviews,* **182**(1), pp. 207-214.

Weiss, L., Donkova-Petrini, V., Caccavelli, L., Balbo, M., Carbonneil, C. and Levy, Y., (2004). Human Immunodeficiency Virus-driven expansion of $CD4^{+}CD25^{+}$ Regulatory T-cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood,* **104**(10), pp. 3249-3256.

West, N., Kost, S., Martin, S., Milne, K., Nelson, B. and Watson, P., (2013). Tumour-infiltrating FoxP3$^+$ lymphocytes are associated with cytotoxic immune responses and good clinical outcome in Oestrogen receptor-negative breast cancer. *British Journal of Cancer,* **108**(1), pp. 155-162.

WHO (2013), Cancer. [Online] Available from: http://www.who.int/mediacentre/factsheets/fs297/en/ (Accessed :21/12/2013).

Wilke, C.M., Wu, K., Zhao, E., Wang, G. and Zou, W., (2010). Prognostic significance of Regulatory T-cells in tumor. *International Journal of Cancer,* **127**(4), pp. 748-758.

Wislez, M., Fleury-feith, J., Rabbe, N., Moreau, J., Cesari, D., Milleron, B., Mayaud, C., Antoine, M., Soler, P. and Cadranel, J., (2001). Tumor-derived granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor prolong the survival of neutrophils infiltrating bronchoalveolar subtype pulmonary adenocarcinoma. *The American Journal of pathology,* **159**(4), pp. 1423-1433.

Wolf, A.M., Wolf, D., Steurer, M., Gastl, G., Gunsilius, E. and Grubeck-Loebenstein, B., (2003). Increase of Regulatory T-cells in the peripheral blood of cancer patients. *Clinical Cancer Research,* **9**(2), pp. 606-612.

Wong, M.T., Jessica, J.Y., Alonso, M.N., Landrigan, A., Cheung, R.K., Engleman, E. and UTZ, P.J., (2010). Regulation of human TH9 differentiation by type I interferons and IL-21. *Immunology and cell biology,* **88**(6), pp. 624-631.

Woo, E.Y., Chu, C.S., Goletz, T.J., Schlienger, K., Yeh, H., Coukos, G., Rubin, S.C., Kaiser, L.R. and June, C.H., (2001). Regulatory CD4$^+$CD25$^+$ T-cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Research,* **61**(12), pp. 4766-4772.

World Bank., (2014) South africa. [Online] Available from: http://data.worldbank.org/country/south-africa (Accessed :23/03/2014).

World Cancer research Fund International, (2013) Oesophageal cancer. [Online] Available from: http://www.wcrf.org/cancer_statistics/data_specific_cancers/oesophageal_cancer_statistics.php (Accessed: 23/03/2014).

Xu, H., Mao, Y., Dai, Y., Wang, Q. and Zhang, X., (2009). $CD4^{+}CD25^{+}$ Regulatory T-cells in patients with advanced gastrointestinal cancer treated with chemotherapy. *Onkologie,* **32**(5), pp. 246-252.

Xu, T., Duan, Q., Wang, G. and Hu, B., (2011). $CD4^{+}$ $CD25^{+}$high Regulatory T-cell numbers and $FoxP3^{+}$ MRNa expression in patients with advanced Esophageal cancer before and after chemotherapy. *Cell Biochemistry and Biophysics,* **61**(2), pp. 389-392.

Yarney, J., Vanderpuye, V. and Clegg Lamptey, J.N., (2008). Hormone receptor and HER‐2 expression in breast cancers among sub‐Saharan african women. *The Breast Journal,* **14**(5), pp. 510-511.

Yoshihara, H., Tanaka, N. and Orita, K., (1986). Suppression of Natural Killer cell activity by surgical stress in cancer patients and the underlying mechanisms. *Acta Medica Okayama,* **40**(2), pp. 113-119.

Yoshioka, T, Miyamoto, M., Cho, Y., Ishikawa, K., Tsuchikawa, T., Kadoya, M., Li, L., Mishra, R., Ichinokawa, K., Shoji, Y., Matsumura, Y., Shichinohe, T., Hirano, S., Shinohara, S., Itoh, T. and Kondo, S., (2008). Infiltrating Regulatory T-cell numbers is not a factor to predict patient's survival in oesophageal squamous cell carcinoma. *British Journal of Cancer,* **98**(7), pp. 1258-1263.

Young, M.R., Newby, M. and Wepsic, H.T., (1987). Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic lewis lung carcinoma tumors. *Cancer research,* **47**(1), pp. 100-105.

Zell, J.A., Tsang, W.Y., Taylor, T.H., Mehta, R.S. and Anton-Culver, H., (2009). Prognostic impact of human epidermal growth factor-like receptor 2 and hormone receptor status in inflammatory breast cancer (IBC): Analysis of 2,014 IBC patient cases from the California cancer registry. *Breast Cancer Res,* **11**(1), pp. r9.

Zhao, D.M., Thornton, A.M., Dipaolo, R.J. and Shevach, E.., (2006). Activated $CD4^{+}CD25^{+}$ T-cells selectively kill B lymphocytes. *Blood,* **107**(10), pp. 3925-3932.

Zheng, S.G., Wang, J., Wang, P., Gray, J.D. and Horwitz, D.A., (2007). IL- 2 is essential for TGF- beta to convert naive $CD4^{+}CD25^{-}$ cells to $CD25^{+}$ $FoxP3^{+}$ regulatory T-cells and for expansion of these cells. *Journal of Immunology (Baltimore, MD.: 1950),* **178**(4), pp. 2018.

Zhou, L., Lopes, J.E., Mark, M.W.C., Ivanov, I.I., Min, R., Victora, G.D., Shen, Y., Du, J., Rubtsov, Y.P., Rudensky, A.Y., Ziegler, S.F and Littman, D.R., (2008). *TGF-*β- induced FoxP3 inhibits TH17 cell differentiation by antagonizing RORgammat function. Nature 453(7192): 236-40

Zhu, Z., Hu, Y., Zhao, Y., Chen, X., Chen, L. and Chen, Y., (2011). Early recurrence and death after esophagectomy in patients with esophageal squamous cell carcinoma. *The Annals of Thoracic Surgery,* **91**(5), pp. 1502-1508.

Zou, W. and Chen, L., (2008). Inhibitory B7-family molecules in the tumour microenvironment. *Nature Reviews Immunology,* **8**(6), pp. 467-477.