

**Biomarkers of HIV associated malignancies and of drug interaction between
anti-retrovirals (ARVs) and chemotherapy**

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Declaration

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ABSTRACT

INTRODUCTION: Altered immune mechanisms play a critical role in the pathogenesis of Non-Hodgkin lymphoma (NHL), as evidenced by increased rates of NHL among HIV+ patients [De Roos et al., 2012; Mellgren et al., 2012].

AIMS: To determine whether biomarkers of B-, T-cell activation, and inflammation are elevated in HIV+NHL patients; and whether cART influences their expression.

METHODS: The expression of CD8+CD38 and FoxP3 were determined by flow cytometry; the serum concentrations of circulating sCD20, sCD23, sCD27, sCD30 and sCD44 were determined by enzyme linked immunosorbent assay (ELISA); and the serum concentrations of circulating IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF- α were determined by meso-scale discovery (MSD) assay in 141 participants consisting of HIV positive NHL (HIV+NHL), HIV negative NHL (NHL); combination antiretroviral treated HIV+ (HIV+ cART), treatment naive HIV+ (cART-naïve HIV+) patients; and healthy controls.

RESULTS: HIV+NHL patients had higher serum concentrations of sCD20 ($p < 0.0001$ and $p = 0.0359$), sCD23 ($p = 0.0192$ and $p < 0.0001$), sCD30 ($p = 0.0052$ and $p < 0.0001$), sCD44 ($p = 0.0014$ and $p < 0.0001$), and IL-4 ($p = 0.0234$ and $p = 0.03360$); and lower expression of FoxP3 ($p < 0.0001$ and $p = 0.0171$) as compared to NHL and HIV+ cART patients. As compared to NHL patients, the serum concentrations of IL-2 ($p = 0.0115$), and TNF- α ($p = 0.0258$) were higher in HIV+NHL patients, while those of IL-1 β ($p = 0.0039$) were significantly lower. HIV+NHL patients had higher expression of CD8+CD38 ($p = 0.0104$), serum concentrations of IFN- γ ($p = 0.0085$), and IL-6 ($p = 0.0265$); and lower serum concentrations of IL-12p70 ($p = 0.0012$) than HIV+ cART

patients. As compared to controls, NHL had higher concentrations of all biomarkers investigated except FoxP3 expression. As compared to HIV+ cART and controls, cART-naïve HIV+ patients had higher concentrations of all biomarkers investigated except sCD23 and FoxP3 expression.

CONCLUSION: Biomarkers of chronic B- and T-cell activation and inflammation are up-regulated in HIV+NHL and the untreated HIV+ state. cART decreases immune activation and inflammation.

OPSOMMING

INLEIDING: Versteurde immuun meganisme speel 'n kritiese rol in die patogeenese van Non-Hodgkin limfoom (NHL), soos aangedui deur verhoogde tempo van NHL onder MIV+ pasiënte [De Roos et al., 2012; Mellgren et al., 2012].

DOELWITTE: Om te bepaal indien biomerkers van B-, T-sel aktivering en inflammasie verhoog is in MIV+NHL pasiënte; en indien kART hul uitdrukking beïnvloed.

METODE: Die uitdrukking van CD8+CD38 en FoxP3 was bepaal deur vloei sitometrie; die serum konsentrasies van sirkulerende sCD20, sCD27, sCD30 en sCD44 was bepaal deur ensiem gekoppelde immuno sorbant toets (ELISA); en die serum konsentrasies van sirkulerende IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 en TNF- α bepaal was deur meso-skaal ontdekking (MSD) toets in 141 deelnemers bestaande uit MIV positiewe NHL (MIV+NHL); MIV negatiewe NHL (NHL), kombinasie antiretrovirale behandeling MIV+ (MIV+ kART); onbehandelde naïewe MIV+ (kART-naïewe MIV+) pasiënte; en gesonde kontroles.

RESULTATE: MIV+NHL pasiente het hoër serum konsentrasies van sCD20 ($p < 0.0001$ en $p = 0.0359$), sCD23 ($p = 0.0192$ en $p < 0.0001$), sCD30 ($p = 0.0052$ en $p < 0.0001$), sCD44 ($p = 0.0014$ en $p < 0.0001$), en IL-4 ($p = 0.0234$ en $p = 0.03360$); en verlaagde uitdrukking van FoxP3 ($p < 0.0001$ en $p = 0.0171$) in vergelyking met NHL en MIV+ kART pasiente. Vergeleke met NHL pasiente, die serum konsentrasies van IL-2 ($p = 0.0115$), en TNF- α ($p = 0.0258$) was hoër in MIV+NHL pasiente, terwyl die van IL-1 β ($p = 0.0039$) beduidend laer was. MIV+NHL pasiente het hoër uitdrukking van CD8+CD38 ($p = 0.0104$), serum konsentrasies van IFN- γ ($p = 0.0085$), en IL-6

($p=0.0265$); en laer serum konsentrasies van IL-12p70 ($p=0.0012$) as MIV+ kART pasiente. Vergeleke met die kontroles, NHL het hoër konsentrasies van al die biomerkers wat geondersoek was behalwe vir FoxP3 uitdrukking. Vergeleke met MIV+ kART en die kontroles, kART-naïewe MIV+ pasiente het 'n hoër konsentrasies van al die biomerkers wat ondersoek was behalwe sCD23 en FoxP3 die uitdrukking.

GEVOLGTREKKING: Biomerkers van kroniese B- en T-sel aktivering en inflammasie is op-gereguleer in MIV+NHL en die onbehandelde MIV+ toestande. kART het immuun aktivering en inflammasie verminder.

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LIST OF ABBREVIATIONS

DESCRIPTION.....	ABBREVIATION
Acquired immunodeficiency syndrome.....	AIDS
AIDS defining cancer.....	ADC
Alanine aminotransferase.....	ALT
Analysis of variance	ANOVA
Antigen presenting cells	APC
Aspartate aminotransferase	AST
Bleomycin, adriamycin, cyclophosphamide, oncovin, dexamethasone	BACOD
Blood urea nitrogen.....	BUN
Burkitt's lymphoma	BL
C reactive protein	CRP
Central nervous system.....	CNS
Clinical stage.....	CS
Cluster of differentiation	CD
Combination antiretroviral therapy	cART
Complete remission.....	CR
Confidence interval.....	CI
Cyclophosphamide, hydroxydaunomycin, oncovin, prednisone	CHOP

Cyclophosphamide, doxorubicin, etoposide	CDE
Cyclophosphamide, vincristine, doxorubicin, methotrexate/ifosfamide, etoposide, cytarabine.....	CODOX-M/IVAC
Cytochrome P	CYP
Deoxyribonucleic Acid.....	DNA
Diffuse large B-cell lymphoma.....	DLBCL
Dose adjusted EPOCH.....	DA-EPOCH
Epstein Barr virus.....	EBV
Ethylenediaminetetraacetic acid.....	EDTA
Etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin.....	EPOCH
Fc receptor III	FcγRIII
Fluorescence in situ hybridization	FISH
Food and drug administration.....	FDA
French hospital data base on HIV	FHDH
Germinal centre.....	GC
Granulocyte colony-stimulating factor	G-CSF
HIV associated Burkitt's lymphoma	HIV-BL
HIV associated Non-Hodgkin lymphoma.....	HIV-NHL
Horseradish peroxidase	HRP
Human herpesvirus herpes virus 8.....	HHV-8

Human immunodeficiency virus	HIV
Human papilloma virus.....	HPV
Immunoglobulin.....	Ig
Interferon gamma.....	IFN- γ
Interleukin	IL
International prognostic index	IPI
Invasive cervical cancer	ICC
Kaposi sarcoma	KS
Lactate dehydrogenase.....	LDH
Major histocompatibility complex.....	MHC
Methotrexate, leucovorin, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone.....	M-BACOD
MicroRNA.....	miRNA
Nanograms per milliliter.....	ng/ml
Natural killer cells	NK
Natural killer	NK
Non Hodgkin lymphoma	NHL
Pathological stage	PS
Phosphate buffered saline.....	PBS
Primary central nervous system lymphoma.....	PCNSL

Primary effusion lymphoma	PEL
Protease inhibitor	PI
Ribonucleic Acid.....	RNA
Rituximab CHOP	R-CHOP
Standard deviation	SD
Surveillance epidemiology and end results	SEER
T helper	T _H
Tetramethylbenzidine	TMB
Tumor necrosis factor alpha.....	TNF- α
United States of America.....	USA
World Health Organization	WHO

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Cancer has been linked with human immunodeficiency virus (HIV) disease from the earliest reports, with clusters of Kaposi sarcoma (KS) cases in young homosexual men [Cottrill et al., 1997]. Since then it has become increasingly recognised that the incidence of cancer among HIV positive (HIV+) individuals is elevated by 4-3500 fold as compared with the general population [Casper 2011]. Cancer is a significant cause of morbidity and mortality in HIV-1 infected patients [Barbaro and Barbarini 2007; Yanik et al., 2013]. HIV disease progressively reduces the effectiveness of the immune system, thus leaving individuals susceptible to opportunistic infections and tumours [Weiss 1993; Sepkowitz 2001]. The increased cancer risk in HIV-1 infected individuals has been associated with a decline in immune function [Biggar et al., 2007]. The mechanism through which lowered immunity increases the risk for cancer is unclear [Mbulaiteye et al., 2003].

However, the proposed mechanisms for the development of cancer in HIV-1 infected patients include impaired immune surveillance resulting in impaired ability to control infections associated with cancer; poor function of the immune cells that normally play a role in destroying cancerous cells; chronic B-cell stimulation, genomic instability, role of oncogenic viruses and dysregulation of cytokine and growth factor production [Taiwo et al., 2010]. Kaposi's sarcoma (KS), non-Hodgkin lymphoma (NHL), and invasive cervical cancer (ICC) occur in excess among HIV+ individuals and are characterized as acquired immunodeficiency syndrome (AIDS) defining cancers (ADCs) [Tirelli et al., 2002; Riedel et al., 2013]. The three ADCs are known

to be associated with viral infections i.e. human herpesvirus 8 (HHV8) for KS, Epstein Barr virus (EBV) for most NHL cases in HIV infected patients, and human papillomavirus (HPV) for ICC [Schulz 2009; Hleyhel et al., 2013; Costagliola 2013]. In the pre-combination antiretroviral therapy (cART) era, the risk for KS development was 3640 fold higher in HIV-1 infected patients, 77 fold higher for NHL development, and 6 fold higher for ICC development as compared to the non-HIV infected population [Grulich et al., 2007]. With the advent of effective cART, the incidence of ADCs has declined [Long et al., 2008; Shiels et al., 2011a], however, the incidence rates remain many times higher in HIV-1 infected patients than those in the HIV negative population [Shiels et al., 2011b; Hleyhel et al., 2013].

In a cohort study of 11 485 HIV-1 infected patients, Yanik and colleagues [2013], reported that the incidence rates for KS and NHL were highest in the first 6 months after cART initiation and plateaued thereafter [Yanik et al., 2013]. Hleyhel and colleagues [2013], in a study of 99 309 HIV-1 infected patients, reported that the incidence of ADCs fell significantly across the calendar period of 2005-2009, but the risk remained constantly higher in HIV-1 infected patients than in the general population. Most epidemiologic studies focused more on KS which is the most common malignancy in HIV setting followed by NHL. Thus the current study will specifically focus on biomarkers associated with the development of HIV associated NHL (HIV+NHL). The prevalence of HIV+NHL increases steadily and the mechanisms leading to its development in HIV-1 infected individuals is poorly understood.

HIV+NHL is a very complex and complicated disease associated with many challenges including drug-drug interactions, thus biomarkers are required to optimize the therapeutic strategies [Kondo 2012]. Biomarkers can offer a great potential for improving management of HIV+NHL by providing its molecular definition, providing information about the course of the disease and predicting response to therapies [Bhatt et al., 2008; Mishra and Verma 2010]. Biomarker studies need to be performed in the target population, e.g. sub-Saharan Africa for HIV+NHL because of the prevalence of HIV in this region. This information is also important in personalizing the treatment care, as different patients may respond differently to the same treatment and in selecting the right drug for the right patient [Vogenberg et al., 2010; de Lecea and Rossbach 2012].

Cancer is a very heterogeneous group of diseases whose pathogenesis, aggressiveness, metastatic potential, and response to treatment can be different among individual patients, making personalised medicine the best solution [Diamandis et al., 2010; Schilsky 2010; Nakagawa 2012]. Immune biomarkers and assays among other functions, also play a vital role in the development of cancer immunotherapy to select the patients expected to respond to immunotherapy before or early after immunotherapy to monitor immune induction following immunotherapy and to evaluate anti-tumor effects early after immunotherapy [Hoos et al., 2010; van der Burg 2011; Kawakami et al., 2012]. Immune biomarkers could also be useful in monitoring other types of NHL therapies.

1.2 HIV associated Non-Hodgkin Lymphoma (HIV+NHL)

Non-Hodgkin Lymphoma (NHL) refers to a heterogeneous group of malignancies of lymphoid origin arising from B lymphocytes (85-90%), and T lymphocytes or natural killer (NK) lymphocytes (10%) [Hiddemann 1995; Hauke and Armitage 2000; Rummel 2010; Shankland et al., 2012]. The exact cause of NHL is not yet known, but it has been previously associated with the presence of EBV [Ometto et al., 1997; Tulpule and Levine 1999; Carbone 2003]. NHL develops from the lymph nodes, but can occur in almost any tissue. It comprises many types, each with distinct epidemiology, aetiology and features (i.e. morphology, immunophenotype and clinical) [Bio Oncology 2012]. These include systemic NHL, primary central nervous system lymphoma (PCNSL) and primary effusion lymphoma (PEL) [Franceschi et al., 1999; Mbulaiteye et al., 2002]. Although all three develop from the lymphocytes, they differ in their presumed origin, mechanisms, pathogenesis, clinical presentation and treatment [Kaplan 1998].

NHL is further classified into low, intermediate, and high grade lymphoma which are based on the treated natural history and survival patterns [Chan 2001]. NHL also develops in immunodeficiency states such as congenital immunodeficiency disorder (i.e. ataxia telangiectasia or Wiskott-Aldrich syndrome), state of pharmacologic immunosuppression (i.e. long term immunosuppressive therapy to prevent transplant rejection or for the management of autoimmune diseases), and the immunodeficient state associated with HIV disease [Hoppe 1987]. NHL has been associated with HIV-1 infections since the beginning of the HIV epidemic [Vishnu and Aboulafia 2012]. This association was first suggested in 1982 after four young men with severe

immunodeficiency were diagnosed with a Burkitt like lymphoma in San Francisco [Ziegler et al., 1982; Ulrickson et al., 2012]. Since then, NHL has been designated as an AIDS defining malignancy. The development of HIV+NHL has been shown to be related to the more advanced age of the patient, low CD4 cell counts and no prior treatment with cART [Matthews et al., 2000]. It is also thought that immune stimulation by the HIV-1 virus and reactivation of previous EBV infection due to defective T-cell surveillance, leads to long term stimulation and proliferation of B lymphocytes resulting in the development of HIV+NHL [Powles et al., 2000].

Furthermore, even in the absence of EBV infection, HIV induces the production of inflammatory cytokines such as interleukin (IL)-6 and IL-10 that are associated with B-cell hyper-stimulation, proliferation, and activation [Masood et al., 1995; Wool 1998]. Systemic NHL is the most common variety of HIV+NHL and it occurs across a broad range of levels of immune function, with a median CD4 T-cell count of approximately $100/\text{mm}^3$ [Kaplan 1998; Kaplan 1997; Levine et al., 1991]. Systemic NHLs constitute about 80% of all HIV associated lymphomas [Goedert et al., 1998], and are generally aggressive and fast growing tumors in HIV-1 infected people [Kalter et al., 1985; Myskowski et al., 1990].

Clinical presentation depends on the site of involvement, natural history of the lymphoma subtype, and presence or absence of B symptoms (weight loss >10% of body weight over 6 months, night sweats, and body temperature >38°C) [Shankland et al., 2012]. Aggressive lymphomas commonly present acutely or sub-acutely with a rapidly growing mass, systemic B symptoms, elevated levels of serum lactate dehydrogenase (LDH) and uric acid [Freedman et al., 2013a]. Indolent lymphomas are often insidious, presenting only with slow growing lymphadenopathy, hepatomegaly, splenomegaly, or cytopenia [Freedman et al., 2013a]. High grade B-cell NHL is the second most common malignancy affecting HIV-1 infected individuals and although studies show a decline in incidence since the introduction of cART, HIV associated lymphomas have increased as a percentage of first AIDS defining illnesses [Lee et al., 2010; Bower et al., 2013].

The most common NHL subtypes arising in HIV associated immunosuppression are diffuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma (BL) [Gloghini et al., 2013]. DLBCL is the most frequent histological subtype occurring in the HIV-1 infected population and accounts for 80% of cases [Lim et al., 2005]. The remaining 20% of HIV+NHL comprise of small non-cleaved cell lymphomas such as BL [Lee et al., 2010]. However, other entities such as plasmablastic lymphoma and B-cell lymphoma, unclassifiable with features intermediate between DLBCL and BL have also been reported in the setting of HIV+NHL [Cesarman 2013].

1.2.1 Diffuse large B-cell lymphoma (DLBCL)

Diffuse large B-cell lymphoma (DLBCL) is defined as a neoplasm of large transformed B-cells (with nuclear diameter more than twice that of a normal lymphocyte) growing in a diffuse or non-follicular pattern [Lowry and Linch 2008], accounting for 30-40% of all adult NHL [de Leval and Hasserjian 2009]. DLBCL is characterized by diffuse nodal architectural effacement or extranodal infiltration by sheets of large cells of B-cell phenotype [Said 2013]. Immunophenotypically, DLBCLs express CD45, and pan-B-cell antigens, such as CD19, CD20, CD45RA, CD79a, and the nuclear transcription factor PAX5 [de Leval and Hasserjian 2009]. The tumour cells usually express a monotypic surface immunoglobulin (Ig), with or without cytoplasmic Ig, usually IgM [de Leval and Hasserjian 2009]. A distinct subtype of DLBCL more commonly seen in HIV-1 infected individuals is plasmablastic lymphoma [Cesarman 2013; Bibas and Castillo 2014; Castillo et al., 2015]. Plasmablastic lymphoma is characterized by a diffuse proliferation of large neoplastic cells, most of which resemble B-cell immunoblasts [Bibas and Castillo 2014].

1.2.2 Burkitt's lymphoma (BL)

Burkitt lymphoma (BL) is an aggressive form of NHL derived from germinal center B-cells [Schmitz et al., 2012]. The tumour consists of high grade, diffuse, small non-cleaved B-cell lymphocytes [Shapira and Peylan-Ramu 1998], and are CD19+/CD20+. BL is one of the most rapidly growing malignancies affecting children and young adults [Levine 2002]. BL is classified into 3 clinical variants i.e. endemic, sporadic, and immunodeficiency associated [Whitten et al., 2012; Said 2013]. Endemic BL occurs in children mostly as extranodal jaw or orbital masses in equatorial Africa and Papua New Guinea [Lowry and Linch 2008; Guech-Ongey et al., 2010]. Sporadic BL is mostly seen in immunocompetent patients, and accounts for high proportion of childhood lymphoma [Lowry and Linch 2008; Said 2013].

Immunodeficiency associated BL is diagnosed in HIV+ individuals, among whom it is the first indication of AIDS onset. HIV associated BL occurs in patients with CD4 T-cell counts >50cells/ μ l and usually presents with nodal disease and bone marrow involvement is commonly seen [Lowry and Linch 2008; Linch 2012; Said 2013]. The classic immunophenotypic profile is that of expression of monotypic IgM (with rare cases of IgG or IgA), CD19, CD20, CD22, CD10, BCL6, CD79a and near 100% expression of Ki-67 [Whitten et al., 2012].

1.2.3 B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL is a heterogenous category that is not considered a distinct entity by the World Health Organization (WHO), but is used as a working classification for cases that may have morphological and genetic features of both DLBCL and BL, but do not fulfil diagnostic criteria for either entity [Aukema et al., 2011; Said 2013; Cesarman 2013; Perry et al., 2013]. This is a temporary category for high grade B-cell lymphomas with a poor clinical outcome [Ota et al., 2014]. It is necessary until better discriminating criteria and more distinct categories of lymphomas are available [Aukema et al., 2011].

1.3 Prevalence of HIV associated Non-Hodgkin Lymphoma (HIV+NHL)

1.3.1 World-wide prevalence of HIV+NHL

An estimated total of 558 340 individuals in the United State (US) population are living with or in remission from NHL [Leukemia and Lymphoma Society 2013]. In 2008, an estimated 355 900 new NHL cases and 191 400 deaths from NHL have occurred [Jemal et al., 2011]. It is expected that approximately 70 800 new cases of NHL will be diagnosed in 2014 and an estimated 18 990 deaths from NHL will occur in US population [American Cancer Society 2014]. The incidence of NHL rises steadily with age, particularly after the age of 30 [Smith 1996]. The median age of patients at diagnosis is 55 years [Hoppe 1987]. However, it may occur even in young children, especially the small non-cleaved cell (Burkitts) and lymphoblastic

lymphomas [Silverberg and Lubera 1987]. From age 20 to 24 years the rate of NHL is about 2.5 cases per 100 000 population; from age 60 to 64 years the rate increases more than 17 times to 44.6 cases per 100 000 population; and from age 80 to 84 years the rate increases more than 47 times to 119.7 cases per 100 000 population [Leukemia and Lymphoma Society 2013]. The age adjusted incidence of NHL rose by 89.5 percent from 1975 to 2010, an average annual percentage increase of 2.6 percent. At ages 20-24 years old, the age specific incidence rates are 3.1 per 100 000 males and 1.9 per 100 000 females; while in ages 60-64 years, the incidence rates are 52.1 per 100 000 males and 37.6 per 100 000 females [Leukemia and Lymphoma Society 2013]. Furthermore, the usual age of patients with HIV+NHL varies in a bimodal distribution pattern i.e. it peaks in adolescence (10-19 years of age) and peaks again at middle age (50-59) [Beral et al., 1991; Levine 2006]. Hingorjo and Syed in [2008], showed a bimodal distribution of NHL with the first peak occurring at 12-13 years and second peak between 52-62 years.

HIV-1 infected individuals have a high risk of developing NHL [Dal Maso and Franceschi 2003]. The cancer data recorded from 11 regions of the United States of America in the Surveillance Epidemiology and End Results (SEER) program, showed that the incidence of NHL (per 100 000) increased gradually from 10.4 in 1973 to 14.5 in 1983 before the onset of HIV epidemic, then more rapidly to peak at 21.1 in 1995 [Eltom et al. 2002; Mbulaiteye et al. 2003]. NHL is regarded as the second most common malignancy associated with HIV-1 infection, with 3-5% of patients presenting with NHL as their first manifestation of AIDS [Wool 1998; Mbulaiteye et al. 2003]. HIV seropositivity increases the risk of developing NHL by

60-165 fold [Bohlius et al. 2009; Vishnu and Aboulafia 2012]. The incidence for high grade NHL is increased by nearly 100 fold in HIV-1 infected individuals [Lyter et al., 1995; Sparano 2001]. It has been reported that NHL is 200-600 times more common in HIV-1 infected individuals as compared with the general population [Aid for AIDS 2010]. Since the beginning of the HIV pandemic, over 25 000 Americans with HIV have been diagnosed with NHL [Ulrickson et al. 2012]. The incidence of NHL increased in most developed countries during the 1990s and has now levelled off or declined in recent years due to the success of cART [Jemal et al. 2011]. The spectrum of malignancies in HIV-1 infected patients has changed in areas where the use of cART is widespread [Deeken et al., 2014].

In a large population of HIV-1 infected patients in the French Hospital Data base on HIV (FHDH), Besson and colleagues [2001], showed that the incidence of systemic NHL has decreased between 1993-1994 and 1997-1998 from 86.0 to 42.9 per 10 000 person years. The incidence in the same cohort was 2.8/1000 person years in 2006 [Bibas and Antinori 2009]. During the calendar period of 2005-2009, the incidence of HIV+NHL fell significantly from 15.4 to 9.1 per 100 000 person years, but the risk remained higher in HIV-1 infected patients than in the general population [Hleyhel et al., 2013]. Furthermore, despite the use of cART, the incidence of NHL still remains relatively high in HIV-1 infected patients and it encompasses a wide variety of disease subtypes for which incidence patterns vary [Engels et al. 2006; Bibas and Antinori 2009; Jemal et al. 2011].

Systemic NHL accounts for the great majority of HIV associated lymphomas and the most common subtypes in HIV+ individuals are DLBCL (approximately 75%) and BL (approximately 25%) [Kaplan et al., 2014]. Approximately 70-90 percent of HIV associated lymphomas are highly aggressive and are almost exclusively the immunoblastic variants of DLBCL and BL [Kaplan et al., 2014]. The relative risk for highly aggressive lymphomas is increased by more than 650 fold for DLBCL and 260 fold for BL as compared with the general population.

In a study by Achenbach and colleagues [2014], it was demonstrated that the incidence of NHL among HIV-1 infected patients receiving cART is higher (171 per 100 000 person years) than that reported in HIV negative individuals (10-20 per 100 000 person years). The availability of cART has enhanced the survival rate of HIV-1 infected individuals; however, the risk of developing lymphoma steadily increases with the duration of HIV-1 infection and advancing immunosuppression [Otieno et al. 2002]. In a study conducted by Mounier and colleagues [2006], it was shown that the overall survival of HIV+NHL patients was significantly higher in the post cART era as compared to the pre cART era (21% versus 37% at 3 years).

1.3.2 Prevalence of HIV+NHL in Sub-Saharan Africa

In most African populations, NHL is rare with the incidence rates well below those seen in Europe and North America although it is often perceived as a common cancer in Africa because it ranks fifth in relative frequency [Sitas et al., 2006; Parkin et al., 2008]. However, there are differences in the incidence of specific subtypes of NHL and their distribution differs by different geographical areas [Anderson et al., 1998]. In addition, there are differences in the racial distribution, e.g. BL is most common in Africa and has seasonal variations [Parkin et al., 2008]. Most NHLs in Africa are of the B-cell type, and clinical series show an excess of high grade lymphomas and a deficit of nodular lymphomas [Sitas et al., 2006; Parkin et al., 2008].

Previous studies have shown that the incidence of NHL in Sub-Saharan Africa did not increase as markedly early in the HIV epidemic when compared to the increase seen in the US HIV+ population [Ulrickson et al., 2012]. However, it has been reported that HIV associated lymphomas are increasing in numerous places in Africa and that the patients are usually diagnosed with late stage disease [Brower 2011]. It has been estimated that approximately 30 000 NHL cases occur in the equatorial belt of Africa each year [De Falco et al., 2013]. Since the beginning of the HIV epidemic, the incidence of NHL has increased by 2-3 fold in some countries, and as much as 13 fold in others [De Falco et al., 2013]. The majority of people (~68%) with HIV live in sub-Saharan Africa, with South Africa having the highest number of cases recorded world-wide [Wiggill et al., 2013; Gopal et al., 2014]. It is estimated that the prevalence of HIV-1 infection in South African adults aged 15-49 is 18-20% and

approximately 170 000-220 000 deaths occurred due to HIV disease [UNAIDS 2014]. Haematological manifestations of HIV including NHL are common and diverse, and can occur at all stages of infection [Opie 2012]. However, accurate epidemiologic, aetiologic and clinical data of HIV+NHL is limited in Sub-Saharan Africa [Wiggill et al., 2013]. Preliminary studies conducted in South Africa suggest that HIV associated lymphomas are increasing in number with increasing HIV prevalence [Wiggill et al., 2013]. Wiggill and colleagues [2011], in a study conducted in Gauteng province, reported that there were 2225 new diagnoses of lymphoproliferative disorders made during 2007-2009 as compared to 1897 cases diagnosed during 200-2006 and more than 90% of all patients diagnosed with high grade B-cell lymphoma were HIV+.

In South African setting, DLBCL and BL represent the most common HIV+NHL [Pather et al., 2013]. In a single institute study conducted in Tygerberg Academic Hospital, Cape Town (Western Cape), over a period of 8 years, Abayomi and colleagues [2011], reported that lymphoma cases increased each year from 2002 to 2005 and remained elevated in both HIV negative and positive patients through to 2009. It was reported that HIV associated lymphomas increased from 5% in 2002 to 37% in 2009 [Abayomi et al., 2011].

1.4 Staging and Treatment of HIV associated NHL (HIV+NHL)

1.4.1 Staging of HIV associated NHL (HIV+NHL)

The Ann Arbor staging system is widely used for the staging of NHL [Hoppe 1987]. Knowledge of the Ann Arbor stage is helpful in determining the appropriate treatment program for patients [Hoppe 1987]. This system divides patients into four stages based on localized disease, multiple sites of disease on one or the other side of the diaphragm, lymphatic disease on both sides of the diaphragm and disseminated extranodal disease [Armitage 1993]. The purpose of a staging system for NHL, for which moderately effective treatments are available, is to identify patients who are more or less likely to respond to treatment [Armitage 1993].

Stage I: refers to involvement of a single lymph node region (I) or of a single extra-lymphatic organ or site (IE); Stage II: refers to the involvement of two or more lymph node regions on the same side of the diaphragm (II) or localized involvement of an extra-lymphatic organ or site and of one or more lymph node regions on the same side of the diaphragm (IIE); Stage III: refers to involvement of lymph node regions on both sides of the diaphragm (III), which may also be accompanied by involvement of the spleen (IIIS) or by localized involvement of an extra-lymphatic organ or site (IIIE) or both (IIISE); Stage IV: refers to diffuse or disseminated involvement of one or more extra-lymphatic organs or tissues, with or without associated lymph node involvement (Table 1.1) [Carbone et al., 1971; Hoppe 1987; Crowther and Lister 1990].

The staging procedure for NHL requires a thorough review of the patient's medical history and a physical assessment including blood work, biopsies, radiologic test, immunophenotyping, and occasionally chromosome testing [O'Brien 2002]. Two imaging modalities have been used in the staging of lymphoma patients i.e. computer tomography and positron emission tomography. Computer tomography (CT) is the principal imaging modality used for patients with lymphoma [Kwee et al., 2008; Delbeke et al., 2009; Wu and Kellokumpu-Lehtinen 2012]. However, CT has several limitations since interpretation of nodal involvement is based only on anatomic criteria of size and shape [Friedberg and Chengazi 2003; Raanani et al., 2006; Delbeke et al., 2009].

2-[Fluorine-18] flouro-2-deoxy-D-glucose positron emission tomography (FDG-PET) which is based on the glycolysis of cancer cells [Delbeke et al., 2009], is a functional imaging modality used for staging and monitoring response to treatment of malignant diseases including lymphoma [Burton et al., 2004; Raanani et al., 2006; Wu and Kellokumpu-Lehtinen 2012]. FDG-PET has higher sensitivity and specificity than CT, however, it requires correlation with anatomical imaging modalities to localize the detected lesion more accurately [Friedberg and Chengazi 2003; Raanani et al., 2006]. Recently, PET/CT systems which enable acquisition of both FDG-PET and CT data at the same setting have been introduced in clinical practice [Raanani et al., 2006; Barrington et al., 2014]. PET/CT systems offer several advantages including shorter image acquisition time, improved lesion localisation and identification and more accurate tumor staging [Raanani et al., 2006]. Currently, the PET/CT system is the standard of care for staging and response assessment in lymphoma patients

[Delbeke et al., 2009; Barrington et al., 2014]. In addition to the staging of NHL, other characteristics such as age, performance status, serum LDH levels, and extra-nodal involvement that have prognostic and therapeutic implications are considered in the treatment and management of NHL [Hauke and Armitage 2000]. The patient's performance score is of importance since a low performance score is associated with decreased tolerance to aggressive treatment and worse outcome [Hauke and Armitage 2000].

Furthermore, pre-treatment evaluation includes CD4 T-cell counts, HIV viral load, hepatitis B and C testing, echocardiogram, creatinine, electrolytes, calcium, phosphate, uric acid, liver function testing and pregnancy test in women [Kaplan 2012]. The absence of generalised symptoms such as fever over 38°C, night sweats, and weight loss of over 10% of body weight in the 6 months preceding diagnosis are denoted by the suffix A, presence of these symptoms is denoted by the suffix B (Table 1.1).

Table 1.1: Ann Arbor staging [Carbone et al., 1971], and Cotswold modification [Crowther and Lister 1990].

Stage	Features
I	Single lymph node region or lymphoid structure
II	Two or more lymph node regions on the same side of the diaphragm
III	Lymph regions or structures on both sides of the diaphragm
IV	Extranodal site(s) beyond that designated E
For all stages	
A	No symptoms
B	Fever (>38°C), drenching sweats, weight loss (10% body weight over 6 months)
For Stages I to III	
E	Single, extranodal site contiguous or proximal to known nodal site
S	In case of spleen involvement
Cotswold modifications	
	Bulky disease: >10cm or 1/3 widening of the mediastinum at T5/6
	Staging should be identified as clinical stage (CS) or pathologic stage (PS)
	Unconfirmed/uncertain complete remission (CR) can be introduced because of the persistent radiologic abnormalities of uncertain significance

1.4.2 Treatment of HIV associated NHL (HIV+NHL)

1.4.2.1 Treatment background

Since the beginning of the HIV epidemic, the treatment of HIV+NHL has been a challenge [Spina and Tirelli 2004]. Earlier in the HIV epidemic, the clinical course of HIV associated lymphoma was dominated by advanced stage disease, concomitant and life threatening opportunistic infections and poor response to treatment [Vishnu and Aboulafia 2012]. In addition, in the management of patients with HIV+NHL, the prognosis was very poor, there was increased haematological toxicity of treatment regimens and a high rate of opportunistic infections [Spina and Tirelli 2004]. As mentioned previously, the clinical course of NHL is much more aggressive in HIV-1 infected patients than in those that are HIV negative [Otieno et al. 2002]. This led to the evaluation of more aggressive and dose dense combination chemotherapy regimens [Otieno et al. 2002].

Efforts to treat patients with HIV associated lymphoma using aggressive and complex chemotherapy regimens led to unacceptable toxicity and early death while low dose chemotherapy regimens yielded modest benefit [Vishnu and Aboulafia 2012]. In the United States, the treatment of HIV associated lymphoma using the CHOP (cyclophosphamide, hydroxydaunomycin (doxorubicin), vincristine (oncovin), and prednisone) regimen achieved complete response rates of 53%, however, these responses were tempered by a rate of relapse of 54% and infectious complications in 42% of the cohort [Ulrickson et al. 2012]. The introduction of cART in the late nineties resulted in great improvement of clinical outcomes and life expectancy for people living with HIV disease [Barbaro and Barbarini 2007; Taiwo et al., 2010]. The

control of HIV viral replication through cART has been accompanied by a reduction in the incidence and progression of HIV associated malignancies, especially KS and NHL [Taiwo et al., 2010]. The concomitant use of cART by patients with HIV associated lymphomas leads to improvement of overall performance status, better response to chemotherapy and survival as compared to the ones not concomitantly using cART [Evison et al., 1999; Besson et al., 2001]. In addition, by combining chemotherapy with cART, the immune function is better maintained in HIV+NHL patients [Powles et al. 2002]. Thus, the benefits of cART include decreased development of HIV associated malignancies, higher CD4 T-cell counts, improved tolerance of full dose of chemotherapy, improved response rates as well as an improved duration of response and survival during treatment of malignancy [Ntekim and Folasire 2010]. This led to the recommendation in the 2005 British HIV guidelines to concomitantly use cART in HIV associated lymphomas [Gazzard 2005].

In addition, the South African HIV guidelines state that all HIV+NHL patients should have concomitant cART, irrespective of their CD4 T-cell counts [Meintjies et al., 2012]. However, there might be more toxicity with the concomitant use of cART, especially in patients with very low CD4 T-cell counts (<100 cells/mm³). In addition, patients with low CD4 T-cell counts often receive antibiotic and antimicrobial prophylaxis to prevent opportunistic infections. The increased incidence and severity of infections in patients with haematological malignancies has led to the development of preventive strategies including prophylaxis with antifungal agents [O'Brien et al., 2003]. Prophylaxis has been associated with the development of adverse reactions and toxicity [Kovacs et al., 2000; O'Brien et al., 2003].

In a study by Little and colleagues [2003], it was shown that patients with CD4 T-cell counts lower than 100 cell/mm^3 that were concomitantly administered DA-EPOCH (dose adjusted-etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin) and cART had increased toxicity and decreased survival rate [Little et al., 2003]. However, dose adjustment with suspension of antiretroviral therapy allowed full delivery of the infused agents, while minimizing clinical and immune toxicity and the treatment was well tolerated [Little et al., 2003]. Treating cancer in HIV-1 infected patients remains a challenge because of drug interactions, compounded side effects, and the potential effect of chemotherapy on CD4 T-cell counts and HIV-1 viral load [Petrella et al., 2004].

Chemotherapy is detrimental to the immune system (especially in the first few months), resulting in accelerated progression of the HIV disease, decline in CD4 T-cell counts and a two-fold increase in opportunistic infections in HIV-1 infected patients diagnosed with cancer [Mackall et al., 1994; Zanussi et al., 1996]. Powles and colleagues [2002], showed a significant decline in CD4 T-cell counts, natural killer cells (CD16/CD56) and B lymphocyte count (CD19 cells) during the first three months of chemotherapy. The CD4 T-cell and natural killer cell counts recovered to pre-treatment levels within one month of finishing chemotherapy [Powles et al. 2002]. It has been reported that many chemotherapeutic agents are cytochrome 3A4 (CYP3A4) substrates, thus there is an increased potential for drug-drug interactions with HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) [Pham and Flexner 2011]. As a result, clinicians are frequently faced with a clinical dilemma of switching to an alternative cART regimen or stopping cART

during chemotherapy [Pham and Flexner 2011]. Furthermore, cancer patients receive a considerable number of drugs during their treatment, including among others, several different cytotoxic agents in multi-drug chemotherapy regimens, hormonal agents, supportive care with anti-emetics, analgesics and anti-infective agents leading to potential drug-drug interactions [Blower et al., 2005]. In addition, in Sub-Saharan Africa many patients use traditional medicines, which also have a potential for drug-herb interactions [Fasinu et al., 2013] The acquisition of prognostic parameters such as biomarkers at initial diagnosis may contribute to implementation of risk based stratification of therapy and may facilitate identification of those who may benefit from early intensive therapy [Tedeschi et al. 2012].

1.4.2.2 Current treatment of HIV associated NHL (HIV+NHL)

NHL responds to most standard of treatments, however, the treatment of HIV+NHL is complicated by the patient's immunocompromised state that also requires specific treatment for HIV disease [Ansell and Armitage 2005; Kaplan et al., 2014]. In addition, the treatment protocols vary according to the type of NHL, however, chemotherapy and radiation therapy are the two principal forms of treatment of NHL [Leukemia and Lymphoma Society 2013]. To treat patients with NHL, the initial pre-treatment evaluation must establish the precise histologic subtype, the extent and site of the disease, and performance status of the patient [Leukemia and Lymphoma Society 2013]. The preferred initial treatment for HIV associated lymphomas has not been defined yet.

Treatment in the immune-competent state involves a combination of modalities including radiation therapy, single agent or combination chemotherapy, immunotherapy, or radioimmuno-conjugate therapy [Leukemia and Lymphoma Society 2013]. Newly diagnosed intermediate or aggressive lymphomas are treated pharmacologically using multi-drug chemotherapy regimen [Flores 2002]. The current first line standard chemotherapy regimen is CHOP [Mehta 2009]. Other dose-adjusted variations such as BACOD (bleomycin, adriamycin, cyclophosphamide, oncovin, dexamethasone), EPOCH (etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin) or some combinations have been attempted in small case series, however, the results were poor, with the median survival of 6 months [Mounier et al., 2009].

In HIV+NHL patients, cART is usually started or modified to control the HIV-1 infection and allow for the administration of chemotherapy and/or radiotherapy [Kaplan et al., 2014]. The choice of therapy is principally determined by the subtype of HIV+NHL and the stage of disease and modifications are made based upon the degree of immunosuppression from HIV disease. The introduction of cART has led to better control of HIV-1 viral replication and improved immune function resulting in better tolerance of chemotherapy, and the incorporation of haematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) into treatment protocols has allowed for the introduction of increasingly myelotoxic regimens. This has allowed conventional chemotherapy regimens used in the HIV negative setting, such as CHOP, to be used as first line treatment in HIV+ patients and outcomes are now similar for those with and without HIV-1 infection [Navarro et al., 2005; Diamond

et al., 2006]. In addition, the concomitant versus sequential administration of cART when applying chemotherapy for HIV+NHL is still a matter of debate but appears to improve overall survival [Weiss et al., 2006; Mounier et al., 2006].

1.4.2.2.1 Treatment of Diffuse large B-cell lymphoma (DLBCL)

Diffuse large B-cell lymphoma (DLBCL) is a very chemosensitive neoplasm and is curable [Cabanillas 2010]. The choice for the first line treatment of DLBCL patients depends upon the extent of disease and on the individual international prognostic index (IPI) score and age [Martelli et al., 2013]. Chemotherapy regimens that have been evaluated for the treatment of DLBCL include CHOP, and continuous infusional regimens such as 96 hours EPOCH and cyclophosphamide, doxorubicin, and etoposide (CDE) [Coiffier 2002]. The CHOP regimen induces complete remission of 40-55 percent with cure rate of approximately 30-35 percent and a three year event free survival rate in DLBCL patients [Fisher et al., 1993; Coiffier 2002].

In HIV negative patients, the standard of care for DLBCL is intravenous CHOP combined with the anti-CD20 monoclonal antibody rituximab (R-CHOP) [Ribera et al., 2008; Sparano et al., 2010; de Witt et al., 2013] The R-CHOP regimen confers two major benefits i.e. a decrease in the number of patients with disease progression during treatment (refractory patients) and a decrease in the number of relapsing patients [Coffier et al., 2010]. The addition of rituximab to CHOP regimen increases the complete response rate and prolongs event free and overall survival rate in DLBCL lymphoma patients [Coiffier 2002; Lowry and Linch 2008]. The rituximab

containing CHOP regimens result in an approximately 10-15 percent overall increase in survival beginning at one year from initiation of therapy with almost no toxicity increase [Sehn et al., 2005; Freedman et al., 2013b]. Furthermore, the study by Coiffier and colleagues [2010], showed a 10 year overall survival (OS) of 43.5% for patients treated with R-CHOP as compared to 27.6% for those treated with CHOP alone [Coiffier et al., 2010].

Although the combination of rituximab with CHOP is well established as first line treatment in HIV negative DLBCL, there remains equipoise regarding safety of rituximab in HIV-1 infected patients with CD4 T-cell counts less than 50 cells per microliter [Kaplan et al., 2005]. Furthermore, in a multicentre randomised study conducted by Kaplan and colleagues [2005], no statistical significant improvement in complete response rate, time to progression, event free, or overall survival in the group treated with rituximab (R-CHOP) when compared with the chemotherapy alone control group (CHOP) could be found.

Current recommendations for first line treatment of DLBCL in HIV-1 infected individuals includes chemotherapy regimens used in HIV negative patients such as CHOP or infusional therapies such as EPOCH, and the gold standard remains to be defined [Bower et al., 2013]. Whether chemotherapy regimens should be concomitantly or sequentially combined with cART still remains a matter of debate. Furthermore, close surveillance may be required for patients with CD4 T-cell count less than 50 cells/mm³.

1.4.2.2.2 Treatment of Burkitt's lymphoma (BL)

Burkitt's lymphoma (BL) is characterised by rapid progression, early haematogenous dissemination and a propensity to spread to the bone marrow and the central nervous system (CNS) [Blay et al., 1991; Shapira and Peylan-Ramu 1998]. In HIV negative patients, BL is a highly curable malignancy if chemotherapy regimens of short duration are combined with CNS penetrating therapy [Bower et al., 2013]. Until recently, patients with HIV associated BL have been treated similar to HIV+ DLBCL patients. However, patients with BL require intensive, frequent multi-agent therapy with adequate CNS prophylaxis [Bishop et al., 2000; Smeland et al., 2004; Freedman et al., 2013c]. The introduction of cART has increased treatment options and improved outcomes for patients with HIV associated BL [Levine 2002]. Approximately 50-80% of patients with BL can be potentially cured with intensive chemotherapy regimens [Levine 2002].

Less intensive regimens such as CHOP used in other NHL subtypes are not adequate therapy as they result in frequent relapses. Lim and colleagues [2005], showed in a retrospective study of 363 patients that the survival of HIV associated BL patients was very poor when treated with CHOP or M-BACOD (methotrexate with leucovorin, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone), despite adjunctive cART. There are 3 main treatment approaches that have been used in patients with BL i.e. intensive, short duration combination chemotherapy such as CODOX-M/IVAC (cyclophosphamide, vincristine, doxorubicin, methotrexate/ifosfamide, etoposide, cytarabine) [Mead et al., 2008]; ALL-like therapy with a stepwise induction, consolidation, and maintenance therapy

lasting at least 2 years from diagnosis such as CALGB 8811 (Cancer and Leukemia Group B study 8811) regimen [Hoelzer et al., 1996; Thomas et al., 1999]; or combination chemotherapy followed by high dose therapy and autologous hematopoietic cell transplantation [Nademanee et al., 1997; van Imhoff et al., 2005; Freedman et al., 2013c]. Alternatively, infusional chemotherapy with dose adjusted EPOCH plus rituximab could be considered for HIV associated BL patients [Sparano et al., 2010; Petrich et al., 2012]. However, there are limited data evaluating the role of rituximab in the treatment of BL. It is now recommended that the first line treatment for BL in HIV+ individuals should include regimens such as CODOX-M/IVAC, DA-EPOCH or similar chemotherapy regimens should be combined with cART [Bower et al., 2013].

1.5 Biomarkers

1.5.1 Definition

Biomarkers are cellular indicators of the physiological and pathophysiological states [Srinivas et al., 2001]. They are objectively measured and evaluated to indicate normal biological processes, pathogenic processes, and pharmacological responses to a therapeutic intervention [Biomarkers Definitions Working Group 2001; Lesko and Atkinson 2001]. Biomarkers can be active genes that are normally inactive, their respective products, and other organic chemicals made by the cell [Srinivas et al., 2001; Mishra and Verma 2010]. In cancer, biomarkers can be normal endogenous products that are produced at a greater rate in cancer cells or the products of newly switched on genes that remained inactive in normal cells [Malati 2007]. For example, the prostate specific antigen (PSA) is present in lower concentrations in the serum of healthy individuals, and is elevated in the presence of prostate cancer [Bhatt et al., 2010; Kilpeläinen et al., 2014].

Biomarkers may include intracellular molecules or proteins that are accessible in body matrices such as tissue cells and body fluids i.e. saliva, serum/plasma, whole blood and urine [Malati 2007; Füzéry et al., 2013]. For example, beta-2 microglobulin (β 2M) is used clinically as a first choice prognostic marker for B-cell leukemia, lymphomas and multiple myeloma [Malati 2007; Nakajima et al., 2014; Yoo et al., 2014]. Wu and colleagues [2014], recently showed that NHL patients with elevated serum levels of β 2M have poor overall survival and higher mortality risk. However, the usefulness of a cancer biomarker depends on its ability to provide early

indication of cancer or its progression and should be easy to detect, and be measurable across populations [Srinivas et al., 2001].

1.5.2 Cancer Biomarker Classification and Utility

It has been well established that a variety of biomarkers are used in risk assessment, early detection, diagnosis, treatment and management of cancer [Verma and Manne 2006; Miaskowski and Aouizerat 2012]. They enable the characterization of patient populations and quantitation of the extent to which drugs reach intended targets, alter proposed pathophysiological mechanisms and achieve clinical outcomes [Frank and Hargreaves 2003]. The most valuable biomarkers are highly sensitive, specific, reproducible and predictable, and the majority of US Food and Drug Administration (FDA) approved cancer biomarkers are serum derived single proteins [Etzioni et al., 2003, Ludwig and Weinstein 2005]. Molecular analyses at the protein, DNA, RNA, or microRNA (miRNA) levels can contribute to the identification of novel tumour subclasses, each with a unique prognostic outcome or response to treatment [Overdevest et al., 2009].

Biomarkers can be classified based on different parameters such as characteristics and function [Sahu et al., 2011; Heckman-Stoddard 2012]. Biomarkers are classified according to their functions i.e. Type 0 biomarkers measure the natural history of a disease and they should correlate over time with known clinical indicators; Type I biomarkers are associated with the effectiveness of pharmacologic agents; and Type II biomarkers also known as surrogate endpoint biomarkers are intended to substitute for clinical endpoints [Rastogi et al., 2008; Sahu et al., 2011; Heckman-

Stoddard 2012]. Current tumour markers may be grouped into a variety of categories including proteins, glycoproteins, oncofetal antigens, hormones, receptors, genetic markers, and RNA molecules [Füzéry et al., 2013].

Cancer biomarkers are also classified into prediction, detection, diagnostic, prognostic, and pharmacodynamics biomarkers [Madu and Lu 2010; Mishra and Verma 2010; Batta et al., 2012]. Prognostic biomarkers are based on the distinguishing features between benign and malignant tumours [Mishra and Verma 2010; Batta et al., 2012]. Predictive biomarkers (also known as response markers) are used exclusively in assessing the effect of administering a specific drug, thus, allowing clinicians to select a set of chemotherapeutic agents which will work best for an individual patient [Mishra and Verma 2010; Batta et al., 2012]. Pharmacodynamic biomarkers are cancer markers utilized in selecting doses of chemotherapeutic agents in a given set of tumor-patient conditions and to assess the imminent treatment effects of a drug [Mishra and Verma 2010; Batta et al., 2012]. Diagnostic markers may be present at any stage during cancer development [Mishra and Verma 2010].

1.5.3 Biomarkers used in clinical diagnosis and prognosis of HIV+NHL

1.5.3.1 Diagnosis

An important step in the diagnosis of NHL is to obtain good quality and adequate samples of tissue by excisional biopsy of an affected lymph node or other mass lesion for assessment of cellular morphology and nodal architecture [Armitage 2007; Steinfort et al., 2010; Kaplan 2012]. After the initial tissue biopsy provides a diagnosis of NHL, the following laboratory tests are performed: complete blood count, white blood cell differential, platelet count, and examination of the peripheral smear for the presence of atypical cells, suggesting peripheral blood and bone marrow involvement; biochemical tests including blood urea nitrogen (BUN), creatinine, alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), LDH, and albumin; serum calcium, electrolytes, and uric acid; serum protein electrophoresis; HIV, hepatitis B and C serology; and beta-2 microglobulin levels (in patients with indolent lymphomas) [Freedman 2013d].

This is followed by pathological evaluations which include flow cytometry or immunohistochemical staining for immunophenotype [Armitage 2007]. For aggressive lymphomas, this includes evaluation of proliferative fraction using Ki67 or MIB-1 staining as a more aggressive regimen may be indicated for high growth fraction tumours [Assem et al., 2001; Kim et al., 2007; Rodig et al., 2008; Kaplan 2012]. Immunophenotypic expression patterns of DLBCL include positivity for various pan B-cell markers such CD19, CD20, CD22, CD79a, PAX-5 and demonstration of immunoglobulin surface light chain restriction by flow cytometry in the majority of cases [Desouki et al., 2010; Sangle et al., 2011]. The presence of

positive PAX-5 immunostaining has been strongly associated with B-cell differentiation as PAX-5 is a B-cell restricted transcription factor [Desouki et al., 2010; Sangle et al., 2011]. Staining for CD10, bcl-6 and MUM-1 are usually routinely performed in order to distinguish Germinal centre (GC) from non-GC DLBCL [Sangle et al., 2011]. In addition, fluorescence in situ hybridization (FISH) analysis for cMYC is performed as translocations involving the cMYC occurs in 10-15% of DLBCL lymphomas and is associated with a worse outcome [Ladanyi et al., 1991]. MYC translocations confer a worse prognosis in patients treated with CHOP and R-CHOP regimens [Horn et al., 2013].

1.5.3.2 Prognosis

It has long been postulated that immune system plays an important role in the etiology of cancer [Beral and Newton 1998]. Immune surveillance is a central mechanism by which cancer development is kept in check [Burnet 1965]. Altered immune mechanisms play a critical role in the pathogenesis of NHL, as evidenced by increased rates of NHL among HIV+ patients, transplant recipients, and autoimmune disease patients [De Roos et al., 2012; Mellgren et al., 2012]. A marked increase in B-cell activation is commonly seen in HIV-1 infection, which is driven by the overproduction of B-cell-stimulatory cytokines, such as IL-6 and IL-10, as well as by stimulation of B-cells by HIV-1 virus and other microbial antigens [Vendrame and Martinez-Maza 2011]. In addition, HIV itself induces the production of inflammatory cytokines that cause B-cell stimulation, proliferation, and activation and the cell lines derived from HIV+NHL have been found to express cytokines including interleukin 6,

10 and tumor necrosis factor β [Masood et al., 1995; Powles et al., 2000]. B-cell activation is characterized by lymphocyte proliferation, class switch recombination, and somatic hypermutation, all of which are prone to resultant errors in DNA that may lead to lymphomagenesis [Breen et al., 2011; De Roos et al., 2012]. Various factors associated with B-cell activation, including B-cell stimulatory cytokines, as well as soluble serum molecules that are associated with B-cell activation, including serum Ig and Ig components such as free light chains, have been seen to be elevated preceding the appearance of HIV+NHL [Landgren et al., 2010].

In a nested case-control study by Breen and colleagues [2011], it was shown that serum concentrations of molecules associated with B-cell activation including IL-6, IL-10, C-reactive protein (CRP), sCD23, sCD27 and sCD30 are elevated for several years preceding the diagnosis of systemic HIV+NHL. In addition, De Roos and colleagues [2012], in a case-control study within the Womens Health Initiative study cohort of 491 cases and 491 controls, showed that women with high serum concentrations of soluble sCD23, sCD27, sCD30, sCD44, and CXCL13 biomarkers were at 2.8-5.5 fold increased risk of B-cell NHL. Furthermore, this was confirmed by Hussain and colleagues [2013], in a nested case-control study of 3768 women where it was shown that elevated concentrations of sCD27, sCD30, CD23, and CXCL13 were associated with subsequent diagnosis of HIV+NHL.

Factors associated with poor clinical outcome and shorter survival in patients with HIV+NHL include CD4 T-cell count $<100/\text{mm}^3$, advanced stage disease (III or IV), age over 35 years, history of injection drug use, elevated LDH (above normal), Eastern Co-operative Oncology Group performance status (ECOG PS) of more than 2 and the involvement of more than 2 extra-nodal sites [Ratner et al., 2001; Ansell and Armitage 2005; Levine 2006].

Matthews and colleagues [2000], in a cohort of 7840 HIV+ patients showed that age, nadir CD4 T-cell count and no prior cART are significantly associated with the development of systemic NHL. In addition, Tedeschi and colleagues [2012], showed that low CD4 and CD8 T-cell count and detectable EBV viremia are three independent prognostic biomarkers that might help in the management of HIV+NHL patients. Furthermore, higher HIV viral load accompanied by lower CD4 count have been associated with the development of HIV+NHL [Guiguet et al., 2009; Engels et al., 2010]. It has been shown that the risk of HIV+NHL rises substantially in patients with HIV RNA levels greater than 100 000 copies/ μl and those with CD4 lymphocyte counts of less than $50 \text{ mm}^3/\mu\text{l}$ [Zoufaly et al., 2009].

CHAPTER TWO

MOTIVATION, HYPOTHESIS, AIMS AND OBJECTIVES

2.1 Motivation

The current study aimed to assess specific biomarkers associated with HIV+NHL. This is very important in South Africa, with its high incidence of HIV disease and HIV+NHL as discussed above. Biomarkers associated with HIV+NHL have not yet been investigated in the South African context. Due to the number of factors including genetic and environmental factors, the profile of these biomarkers could differ from those seen in other populations. The incidence of cancer differs by countries, i.e. one cancer tends to be more common in one country while its incidence is lower in another [Alberts et al., 2002].

Several environmental and behavioural factors alter the risk of developing NHL [Hartge and Smith 2007]. In addition, sociodemographic factors, medical history of immunological disorders, as well as several occupational and environmental chemical exposures such as benzene, other organic solvents, and pesticides have been shown to be risk factors for NHL [Fabbro-Peray et al., 2001; Hartge and Smith 2007; Bassig et al., 2012]. These environmental factors could alter the genomic lesions in B-cells that are not normally lethal, stimulate immune hyperactivation and inflammation leading to the development and progression of NHL [Skibola et al., 2007]. The present study could provide important information on biomarkers of HIV+NHL, which might assist in better understanding of the pathogenesis of NHL in

HIV-1 infected individuals. The outcome of this study will add to the current knowledge available on HIV+NHL.

2.2 Hypothesis

- There is an association between specific biomarkers of B- and T-cell activation, and inflammatory markers and HIV+NHL.
- There is an association between HIV-1 infection, use of cART and HIV+NHL.
- cART decreases immune activation and inflammation in HIV+NHL patients.

2.3 Aims

- Primary aim:
 - To determine whether selected biomarkers of B-, T-cell activation and inflammatory markers are associated with the presence of NHL in HIV-1 infected patients.
- Secondary aims:
 - To determine whether biomarkers of B- and T-cell activation are elevated in HIV+NHL patients.
 - To determine whether inflammatory markers are elevated in HIV+NHL patients.
 - To determine whether cART use has an effect on the biomarker profiles.

Biomarkers for potential drug-drug interactions between cART and chemotherapeutic agents used for HIV+NHL have been included in the initial proposal, but at a later stage it was decided to study biomarkers of HIV+NHL in more detail. The reason was to focus more on a large number of immunological biomarkers in order to provide a better understanding of the potential changes in HIV associated NHL patients, instead of the initially planned broader scope that included biomarkers of drug-drug interactions.

Although biomarkers of potential drug-drug interactions were not explored in the current study, a thorough literature review was conducted on drug-drug interactions in HIV associated malignancies and a review manuscript was written and published in an accredited peer reviewed journal [Flepisi et al., 2014a]. In addition, a thorough literature search on biomarkers of HIV associated malignancies was conducted and published in an accredited peer reviewed journal [Flepisi et al., 2014b].

2.4 Objectives

- Primary objective:
 - To determine the serum concentrations of biomarkers of B-cell activation, inflammation; and the levels of expression of biomarkers of T-cell activation and regulation in all study participants and to compare these to control populations.
- Secondary objectives:
 - To determine the levels of expression of selected biomarkers of T-cell activation and regulation in all study participants and to compare these to control populations.
 - To determine the concentrations of circulating biomarkers of B-cell activation in the serum in all study participants and to compare these to control populations.
 - To determine the concentrations of circulating inflammatory markers in the serum in all study participants and to compare these to control populations.

CHAPTER THREE

STUDY DESIGN AND POPULATION GROUPS

3.1 Study design

- The study is an observational, cross-sectional hospital based clinical study

3.2 Study population

3.2.1 Inclusion criteria

- Patients diagnosed with HIV disease with a nadir CD4 T-cell count of ≤ 350 cells/mm³, patients with HIV+NHL and a healthy control group were included in this study.
- HIV+ patients were screened prior to inclusion into the study.
- The control group were participants with and without NHL but HIV sero-negative.
- HIV test and counselling were done on healthy control population prior to the enrolment into the study.
- All NHL including HIV+NHL patients had DLBCL subtype and were on similar treatment.
- The duration of cART treatment was from three months and above.
- The duration of chemotherapy was from 2 cycles and above.
- Participants were assigned to one of 5 population groups at the first visit.
- The population groups are presented in figure 3.1, further details can be found in *appendix I*.

- The main group of the current study was HIV+ patients diagnosed with NHL receiving cART, CHOP and supportive drugs.
- The HIV+ patients that are not on cART were mainly recruited on their first visit to the hospital.
- Participants were 18 years old and above.

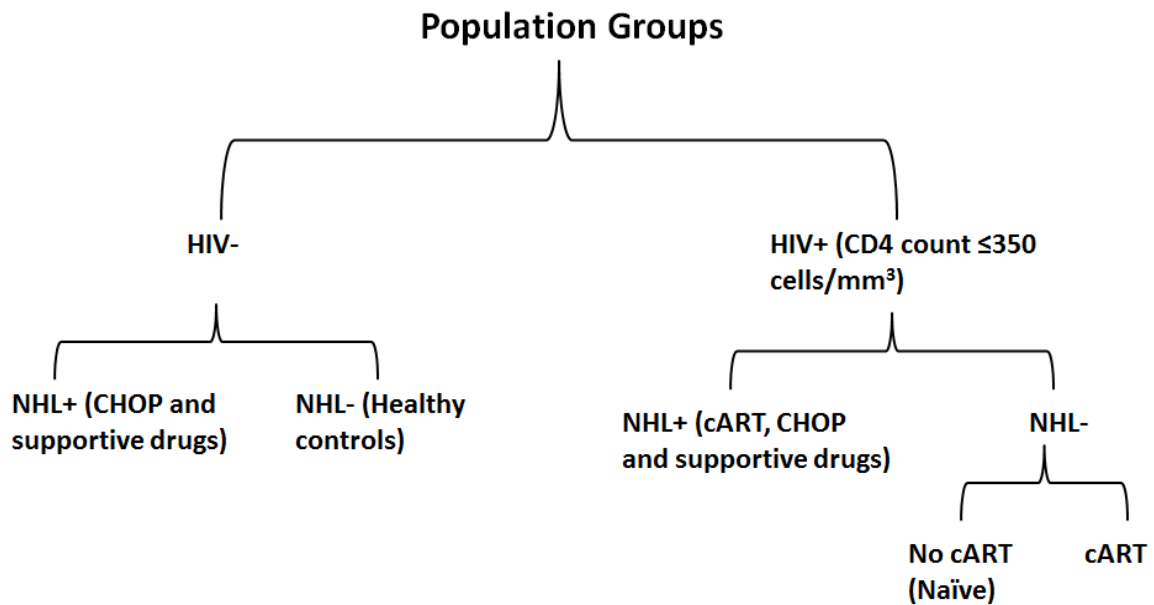


Figure 3.1 *Population groups*: HIV-human immunodeficiency virus; NHL-non-Hodgkin lymphoma; cART-combination antiretroviral therapy; CHOP-cyclophosphamide, doxorubicin, vincristine and prednisone.

3.2.2 Exclusion criteria

- Participants who were not willing to provide information on their environmental background and their habits such as smoking or alcohol usage.
- Participants who were not willing or able to sign the informed consent forms.
- Participants whose medical condition was such as to make the drawing of blood inadvisable such as patients with anaemia i.e. haemoglobin levels of less than 10.0 g/dL (defined according to the division of acquired immunodeficiency syndrome (DAIDS) toxicity tables) [DAIDS 2013].
- Participants who were taking immune suppressants or who were on medication for autoimmune diseases.
- Participants with mental condition rendering them unable to understand the nature, scope, and consequences of the study.
- Participants less than 18 years old.

3.2.3 Sample size

The sample size estimation was performed based on the use of serum CD44 as the primary outcome measure resulting in a difference in means of 70ng/ml with an assumed standard deviation of 130ng/ml. In 194 NHL patients, Ristamaki and colleagues [1997] showed that a high sCD44 level at diagnosis is associated with a high tumour burden, poor response to treatment and unfavourable outcome. In addition, Vendrame and colleagues [2014], in 179 HIV+NHL patients, showed that long term chronic immune activation driven by macrophage produced cytokines precedes the development of NHL in HIV+ individuals.

Using a one-way analysis of variance (ANOVA), a sample size of 28 participants per group achieves 81% power to detect an effect size of 0.2638 among the groups using an F-test with a 0.05 significance level. (Calculations performed by Justin Harvey, Centre for Statistical Consultation, Stellenbosch University)

The recruitment of participants was conducted from October 2012 to February 2014. A total of 141 participants (61 males and 80 females) were recruited in the present study. Participants consisted of 31 HIV+NHL, 34 NHL, 32 HIV+ cART patients and 16 controls (Table 3.1). Although there were only 16 controls, statistical significance between the groups was observed. The mean age of all participants was 40. There were 53 black, 61 coloured, and 27 white participants. 48 participants were active smokers, while 93 were non-smokers. The mean HIV viral load was 4 905 copies/ml in HIV+NHL, 1 044 copies/ml in HIV+ cART, and 19 008 copies/ml in cART naïve HIV+ patients. The mean duration of cART treatment was 24 months, while the mean duration of chemotherapy was 3 cycles.

Table 3.1: Participant characteristics

Characteristics	HIV+NHL	NHL	HIV+ cART	cART-naïve HIV+	CONTROLS
Total Sample size	31	34	32	28	16
Age (mean)	40	50	40	35	34
Gender:					
Males	13	14	13	14	7
Females	18	20	19	14	9
Race:					
Black	15	6	11	17	4
Coloured	10	21	16	9	5
White	6	7	5	2	7
Smoking Status:					
Smoking	9	15	11	8	5
Not smoking	22	19	21	20	11
HIV viral load (Copies/ml) (mean)	4 905		1 044	19 008	

3.2.4 Sample collection

In the current study, blood samples (1 X 4.5 ml clotted blood and 1 X 4.5 ml ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood) from all participants were utilized. Blood was withdrawn using a 10 ml syringe and collected in evacuated tubes: the clotted samples were centrifuged and the serum was separated and stored at -80°C.

The fresh anti-coagulated blood samples were processed as soon as possible (not longer than 24 hours in storage) by staining using monoclonal antibodies and analysed using flow cytometry (section 4.3.2.2).

3.3 Statistical analysis

The data obtained was captured using Microsoft Excel and Graph pad prism version 5 was used to analyse the data. The statistical analysis was conducted by the statistician. The data was transformed using natural logarithm (ln) prior to the analysis. The one-way analysis of variance (ANOVA) with Bartlett's test for equal variances was conducted. The analysis of the primary objective was performed by using a Kruskal Wallis with Dunn's post hoc test.

The study populations were regarded as the independent variables and the specific marker value was regarded as the dependent variable. Relationships between two continuous variables was analysed with logistic regression analysis and the strength of the relationship measured with the Pearson correlation, or Spearman correlation. Multivariate analysis was conducted to assess biomarkers that were independently associated with HIV+NHL. A p-value of $p < 0.05$ represented statistical significance in hypothesis testing and 95% confidence intervals was used to describe the estimation of unknown parameters.

3.4 Ethical considerations

This study was approved by the Health Research Ethics Committee at Stellenbosch University (N12/03/015) and University of Cape Town (076/2013). All participants completed and signed the informed consent forms, which were available in English, Afrikaans and Xhosa. There was a separate genetic consent form to be signed by participants. Participants did not receive feedback on their biomarker levels, because of the low predictive value of these biomarkers for the disease progression in individual patients. Patient details and contact information was kept confidential and data was presented anonymously. The study was carried out in accordance with the Helsinki Declaration [World Medical Association Declaration of Helsinki 2008]. Permission was obtained from the Tygerberg Hospital and Groote Schuur Hospital Board to gain access to patients' folders.

CHAPTER FOUR

T, B, NK AND NKT-CELLS

4.1 Introduction

Lymphocytes are white blood cells generated by the immune system to protect the body against cancerous cells, pathogens, and foreign matter [Bailey 2014; Veillette et al., 2014]. They are responsible for the determination of specific immune response against several foreign substances [Moore et al., 2001; Humphrey and Perdue 2014]. Lymphocytes originate from stem cells in the bone marrow and they either travel to the thymus where they multiply and differentiate into T lymphocytes (T-cells) or remain in the bone marrow (B lymphocytes/cells) [Humphrey and Perdue 2014].

T-cells make up approximately 22-30% of circulating lymphocytes, while B-cells represent 7-10% [Spaner and Bahlo 2011]. There are three main types of lymphocytes i.e. T-cells, B-cells, and natural killer cells and each type has distinctive biochemical and functional characteristics [Martini et al., 2014]. T-cells mediate cellular immunity, while B-cells mediate humoral immunity and they both provide adaptive immunity, which work in close collaboration with the innate immune system [Luckheeram et al., 2012].

4.1.1 T lymphocytes (T–cells)

T lymphocytes (T–cells) arise from the bone marrow stem cells, then migrate to the thymus where in the course of maturation their specific character and subsequent role are determined [Israels and Israels 1999; Bailey 2014]. They are responsible for cell mediated immunity and they can eradicate malignant cells [Jin et al., 2011]. T-cells recognize antigens through a unique antigen specific $\alpha\beta$ -T-cell receptor (TCR), promote the elimination of the targeted antigen (effector function) and amplify the attack of the antigen by recruiting other components of the immune response (helper function) [Hoyos et al., 2012]. There are three major classes of T-cells i.e. cytotoxic T-cells, helper T-cells, and regulatory T-cells [Jin et al., 2011].

4.1.1.1 Helper T-cells

Helper T-cells are a special subpopulation of CD4+ T-cells that stimulate other cells of the immune system to mount immune responses by causing cell activation or the secretion of cytokines [Ibelgaufts 2012]. Helper T-cells are regarded as the most important cells in adaptive immunity, as they orchestrate all adaptive immune responses [Alberts et al., 2002; Zhu and Paul 2008]. They are critical in co-ordinating the activity of the immune response [Alberts et al., 2002; Zhu and Paul 2008]. CD4+ T-cells stimulate B-cells to produce antibodies, induce macrophages to develop enhanced microbicidal activity, recruit neutrophils, eosinophils, and basophils to sites of infection and inflammation, and through their production of cytokines and chemokines orchestrate the full immune responses [Alberts et al., 2002; Zhu and Paul 2008].

Helper T-cells become activated when they are presented with peptide antigen by major histocompatibility class II (MHC II) molecules, which are expressed on the surface of antigen presenting cells (APC) [Israels and Israels 1999]. Following activation, naïve CD4 T-cells differentiate into functionally distinct T helper subsets (T_H1 , T_H2 or T_H17 effector cells), each regulated by key transcription factors and producing cytokines to perform specific biological functions [Alberts et al., 2002; Zhou et al., 2009; Califano et al., 2014]. The differentiation decision is governed predominantly by the cytokines in the microenvironment and, to some extent, by the strength of the interaction of the T-cell antigen receptor with antigen [Zhou et al., 2009]. These effector cells can be distinguished by the cytokines they secrete i.e. T_H1 cells secrete among others IL-1, interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α); T_H2 cells secrete interleukin 4, 10, and 13; and T_H17 cells produce IL-17, and IL-22 [Alberts et al., 2002; Coussen and Werb 2002; Zhou et al., 2009; Califano et al., 2014]. T_H1 are also known to activate cytotoxic T-cells, while T_H2 stimulates B-cells.

4.1.1.2 Cytotoxic T-cells

Cytotoxic CD8⁺ T-cells are antigen specific effector cells of the immune system with the ability to lyse target cells such as virus infected cells, tumor cells, or even parasites in a contact dependent manner [Groscurth and Filgueira 1998; Israels and Israels 1999]. Cytotoxic T-lymphocytes destroy cells infected with intracellular pathogens via T-cell receptor (TCR) mediated recognition of class I human histocompatibility linked leukocyte antigens (HLA-1) [Parsons et al., 2010]. They are crucial for protection against primary infection with non or poorly cytopathic viruses and other intracellular pathogens [Maher and Davies 2004; Schurch et al., 2014].

Upon activation by antigen presenting cells (MHC I), naïve CD8⁺ T-cells undergo clonal expansion, migrate to sites of infection, and kill infected target cells via secretion of perforin and granzymes or Fas ligand-Fas interaction [Andersen et al., 2006; Schurch et al., 2014]. Cytotoxic T-cells are responsible for anti-tumor activity and the presence of T-cells in both numbers and functionality is a prerequisite for the immune system to attack cancer cells [Aerts and Hegmans 2013]. It is recognized that IFN- γ producing CD4⁺ T_H1 cells and CD8⁺ T-cells play an important role in inhibiting and killing tumor cells and impeding tumor growth [Zamarron and Chen 2011].

4.1.2 B-cells

B lymphocytes (B-cells) are lymphocytes that have developed and matured in the bone marrow [Janeway et al., 2001; Silverthorn 2004; Tobon et al., 2013]. A mature B-cell is activated when it encounters antigen that expresses epitopes that are recognized by surface immunoglobulins with the aid of helper T-cells [Moore et al., 2001; Alberts et al., 2002]. B-cells are antibody producing precursor cells which when activated, undergo clonal selection and differentiation to produce effector cells known as plasma cells and memory cells [Wahl and Rosenstreich 1976; Israels and Israels 1999; Humphrey and Perdue 2014; Schnurman 2014].

Plasma cells generate large amount (approximately 2000 molecules per second) of soluble antibodies or immunoglobulins which are released into the circulation [Alberts et al., 2002; Humphrey and Perdue 2014; Schnurman 2014]. The antibodies produced by plasma cells are specific for a specific antigen [Yanaba et al., 2008]. Antibodies defend against infection by inactivating viruses and microbial toxins, and recruiting the complement system and various types of leukocytes to destroy the invading pathogens [Alberts et al., 2002]. In addition, they have been considered as positive regulators of immune responses and central contributors to the pathogenesis of immune related diseases such as autoimmune disease [Yanaba et al., 2008; Mauri and Bosma 2012].

CD19 is a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily, that plays a role in the antigen-independent development as well as the immunoglobulin induced activation of B-cells [Wang et al., 2012; Raufi et al., 2013]. CD19 is expressed in normal and neoplastic B-cells, as well as follicular dendritic

cells [Craig 2007; Wang et al., 2012]. The expression of CD19 is restricted to B-cells and is present throughout B-cell maturation [Craig 2007]. Its expression is 3-fold higher in mature B-cells than that found in immature B-cells [Carter et al., 2002; Diamant et al., 2005]. CD19 is a B-cell restricted signalling molecule that functions as a positive regulator of B-cell receptor [Diamant et al., 2005; Otero and Rickert 2003]. CD19 is expressed by most B-cell malignancies and is regarded as one of the most reliable surface marker for B-cells [Craig 2007; Wang et al., 2012].

4.1.3 Natural killer (NK) cells

Natural killer (NK) cells are cytolytic and cytokine producing effector cells of the innate immune system and they represent the first line of defence against virally infected and transformed malignant cells [Vivier et al., 2004; Wendel et al., 2008; Przewoznik et al., 2012]. NK-cells function similarly to cytotoxic T-cells [Bailey 2014]. However, unlike T-cells, their response to an antigen is non-specific and they do not have T-cell receptors [Jost and Artfeld 2013; Bailey 2014]. In addition, NK-cells do not directly attack invading micro-organisms but instead destroy the body's own cells that have been infected with a virus or that have become cancerous [Humphrey and Perdue 2014]. Their effector functions include release of cytotoxic granules containing perforin and granzymes and induction of death receptor mediated apoptosis resulting in direct killing of target cells [Vivier et al., 2004; Wendel et al., 2008; Przewoznik et al., 2012].

In addition, NK-cells produce the pleiotropic cytokine IFN- γ that is important for activation of APCs and the induction of T_H1 responses [Wendel et al., 2008; Vivier et al., 2008; Przewoznik et al., 2012]. In humans, NK-cells express CD56, a surface antigen that has also been found on a subset of T-cells and CD16, an Fc receptor III (Fc γ RIII) involved in antibody dependent cellular cytotoxicity that is also expressed on a subset of CD3⁺/CD8⁺ T-cells [Cooper et al., 2001; Brunetta et al., 2010; Romee et al., 2013]. The majority (90%) of circulating NK-cells have low density expression of CD56 phenotype and express high levels of CD16 and they are CD3 negative [Cooper et al., 2001; Brunetta et al., 2010; Bostik et al., 2010; Romee et al., 2013].

4.1.4 Natural killer T (NKT) cells

Natural killer T (NKT) cells are a group of T-cells that express surface markers associated with cells of the NK-cell lineage [Biron and Brossay 2001; Bendelac et al., 2007; Van Kaer et al., 2013]. NKT-cells are therefore a subset of T-cell lineage expressing NK lineage receptors in addition to semi-invariant CD1d restricted $\alpha\beta$ T-cell receptors [Bobryshev 2005; Tupin and Kronenberg 2006; Bendelac et al., 2007; Cianferoni 2014]. They are important regulators in both innate and adaptive immunity [Terabe and Berzofsky 2008; Fang et al., 2010]. NKT-cells play important and multifaceted roles in immune regulation, tumor rejection and resistance to a variety of viral, bacterial, and parasitic pathogens through their rapid secretion of immunoregulatory cytokines and potent cytotoxicity [Eger et al., 2006].

NKT-cells can be activated in both antigen-dependent and independent manners and respond with robust T_H1 and T_H2 cytokine production [Juno et al., 2012]. Upon activation, NKT-cells rapidly release cytokines such as IFN- γ , IL-4, IL-13, and IL-17 [Konek et al., 2012]. In addition, NKT-cells have been shown to activate B-cells, resulting in increased Ig secretion and response to antibodies [Galli et al., 2003]. Furthermore, NKT-cells have been implicated in a wide range of disease conditions including tumors, auto-immune diseases, atherosclerosis, allergy and infections [Bendelac et al., 2007].

4.2 Specific Aims

The aim of this study was to determine

- The numbers of CD4+, CD8+, CD19+, NK and NKT-cells in HIV+NHL patients and control populations.
- Whether cART has an influence on the numbers of these cells.

4.3 Materials and Methods

4.3.1 Materials

The following antibodies, materials and reagents were purchased from the Scientific Group Pty Ltd supplier of Becton Dickinson antibodies: Multitest CD3/CD8/CD45/CD4 (Catalogue number: BD/342417), Multitest CD3/CD16+CD56/CD45/CD19 (Catalogue number: BD/342446), fluorescence-activated cell sorting (FACS) Lysing Solution (Catalogue number: BD/349202), Trucount Tubes (Catalogue number: BD/340334), Trucount Controls (Catalogue number: BD/340335), Ethylenediaminetetraacetic acid (EDTA) tube (Catalogue number: BD367864), and Becton-Dickinson (BD) falcon tubes (Catalogue number: BD/352054). Phosphate buffered saline (PBS) (Catalogue number: P5368-10PAK) was purchased from Sigma-Aldrich SA PTY LTD.

4.3.2 Methods

4.3.2.1 Sample preparation

Blood samples were taken using EDTA anti-coagulated vacutainer tubes. The blood vials were requested to be full draws and kept in a plastic bag at room temperature. Whole blood samples were stained and analysed immediately post-staining using flow cytometry.

4.3.2.2 Flow Cytometry

Flow cytometry is a technology that simultaneously measures and then analyses the physical and chemical properties of cells or cellular components, as they flow in a fluid stream through a beam of light [Becton and Dickinson 2000]. Flow cytometry has been previously used in the diagnosis and evaluation of B-cell lymphoid malignancies including NHL [Craig 2007; de Tute 2011; Gunduz et al., 2013]. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity [Becton and Dickinson 2000]. These characteristics are determined using an optical to electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence [Becton and Dickinson 2000]. A flow cytometer is made up of three main systems i.e.

- i) Fluidic system which transports particles in a stream to the laser beam for interrogation.
- ii) Optic system which consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to appropriate detectors.
- iii) Electronic system which converts the detected light signals into electronic signals that can be processed by the computer.

In the flow cytometer, particles are carried to the laser intercept in a fluid stream [Becton and Dickinson 2000]. Any suspended particle of cell from 0.2-150 micrometers in size is suitable for analysis [Becton and Dickinson 2000]. When particles pass through the laser intercept, they scatter laser light and any fluorescent molecules present on the particle fluoresce [Becton and Dickinson 2000]. The

scattered and fluorescent light is collected by appropriately positioned lenses [Becton and Dickinson 2000]. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors and the detectors produce electronic signals proportional to the optical signals striking them [Becton and Dickinson 2000].

Immunophenotyping by flow cytometry is a well-established technique used in many research and clinical environments [Festin et al., 1994]. Flow cytometry allows researchers and clinicians to perform complex analysis quickly and efficiently by analysing several parameters simultaneously [Becton and Dickinson 2000]. The amount of information obtained from a single sample can be further expanded by using multiple fluorescent reagents [Becton and Dickinson 2000]. Multicolor flow cytometry enables more data to be gathered about a sample in a shorter amount of time, giving researchers not only enhanced efficiency and high quality data, but also more data from lower sample volume [Becton and Dickinson 2000].

4.3.2.3 Protocol

To determine the expression of T, B, NK and NKT-cells, trucount tubes were used. Trucount tubes were labelled for T, B, NK, and NKT-cells for each sample in duplicate, and CD chek standards (Low and Normal). Twenty μ l of the diluted (1:3) (*Appendix II*) multi-test CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC and CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC antibodies were added into T, B, NK, and NKT-cell tubes. Fifty μ l of the whole blood samples and CD chek controls were added into respective tubes. Tubes were vortexed gently and samples were incubated at room temperature (20-25°C) in the dark cupboard for 15 minutes. Following the incubation, 450 μ l FACS lysing solution was added into all tubes. Tubes were vortexed gently and samples were incubated at room temperature in the dark cupboard for 15 minutes. The samples were analysed using FACS Canto DIVA software.

4.4 Results

4.4.1 T-cells

4.4.1.1 CD4+ T-cells

The numbers of CD4+ T-cells were significantly lower in HIV positive NHL (HIV+NHL) patients as compared to both HIV negative NHL (298 ± 218 vs 537 ± 375 ; $p=0.0035$) patients and HIV positive patients on a cART regimen (HIV+ cART) (298 ± 218 vs 417 ± 260 ; $p= 0.0401$) (figure 4.1). However, NHL had significantly lower CD4+ T-cell counts than controls (537 ± 375 vs 1101 ± 284 ; $p<0.0001$). HIV+ cART individuals had increased numbers of CD4+ T-cells than cART-naïve HIV+ patients (417 ± 260 vs 238 ± 153 ; $p=0.0060$). In addition, cART-naïve HIV+ patients had lower CD4+ T-cell counts than healthy controls (238 ± 153 vs 1101 ± 284 ; $p<0.0001$).

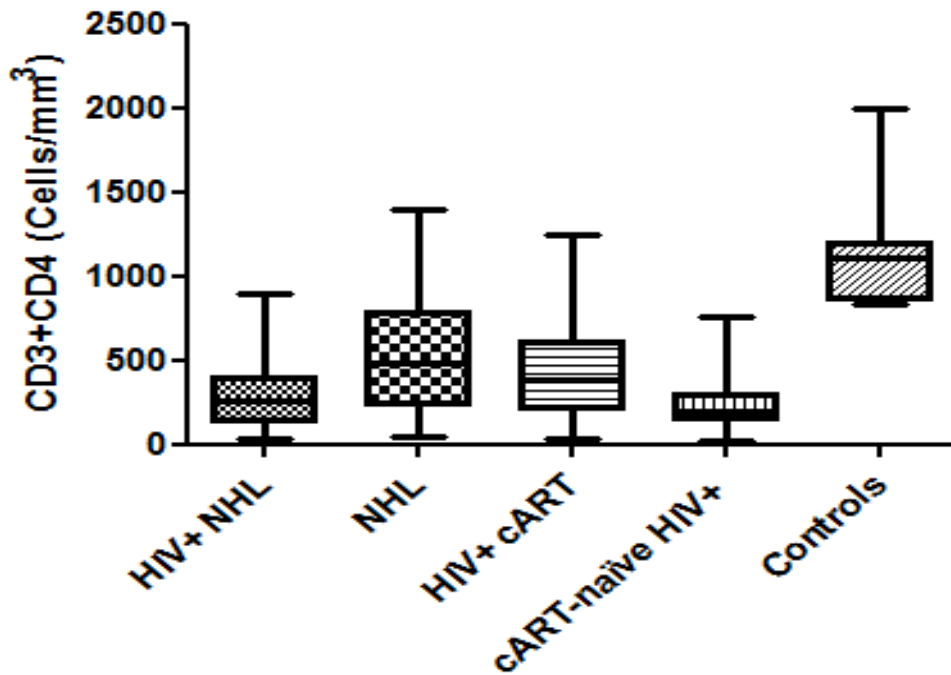


Figure 4.1 *CD3+CD4+ T-cells*. HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

4.4.1.2 CD8+ T-cells

The numbers of CD8+ T-cells were significantly increased in HIV positive NHL (HIV+NHL) patients as compared to HIV positive patients on a cART regimen (HIV+ cART) (701 ± 377 vs 544 ± 271 ; $p=0.0368$), while there was no significant difference between HIV+NHL and NHL patients (figure 4.2). However, NHL patients had significantly higher numbers of CD8+ T-cells than healthy controls (589 ± 503 vs 426 ± 124 ; $p=0.0114$). HIV+ cART patients had significantly lower numbers of CD8+ T-cells as compared to cART-naïve HIV+ patients (544 ± 271 vs 811 ± 403 ; $p=0.0077$). In addition, cART-naïve HIV+ patients had increased numbers of CD8+ T-cells than healthy controls (811 ± 403 vs 426 ± 124 ; $p=0.0002$).

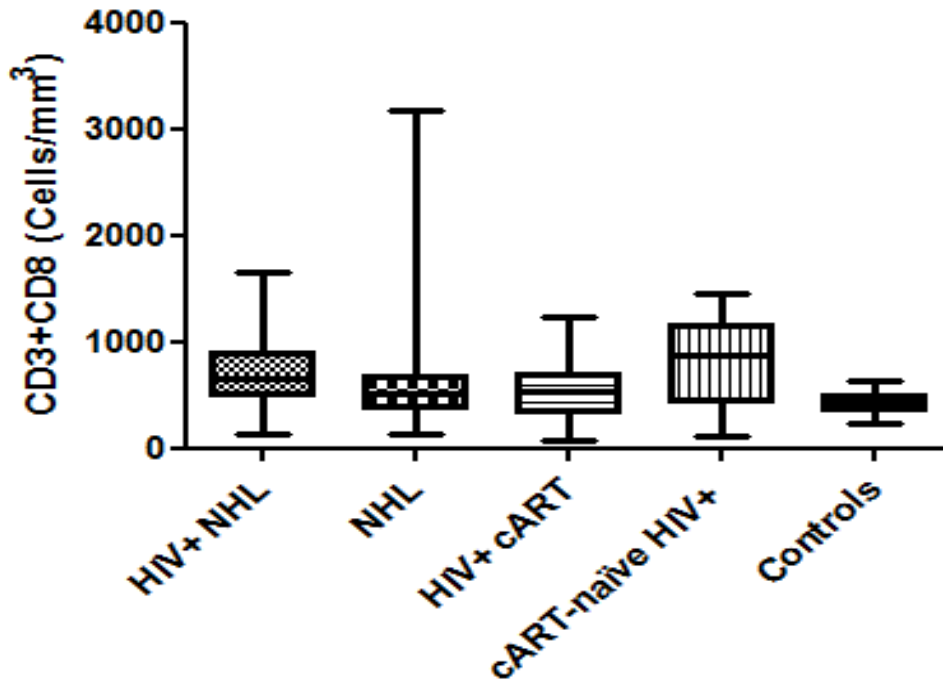


Figure 4.2 *CD3+CD8+ T-cells*. HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

4.4.2 CD19+ B-cells

There was no significant difference in the numbers of CD19+ B-cells when HIV positive NHL (HIV+NHL) patients were compared to both HIV negative NHL patients and HIV positive patients on a cART regimen (HIV+ cART) (figure 4.3). The numbers of CD19+ B-cells were significantly lower in NHL patients as compared to the controls (277 ± 262 vs 358 ± 138 ; $p=0.0021$). The numbers of CD19+ B-cells were significantly increased in HIV+ cART patients as compared to cART-naïve HIV+ patients (262 ± 189 vs 108 ± 73 ; $p<0.0001$). In addition, cART-naïve HIV+ patients had significantly lower numbers of CD19+ B-cells than the controls (108 ± 73 vs 358 ± 138 ; $p<0.0001$).

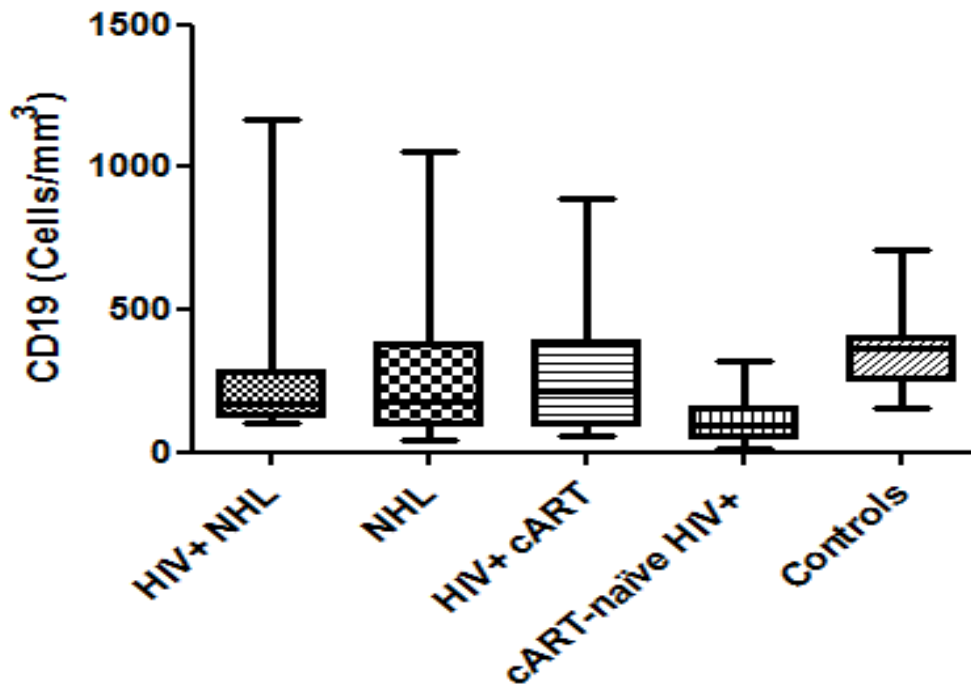


Figure 4.3 *CD19+ B-cells*. HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

4.4.3 Natural killer (NK) cells

The numbers of natural killer (NK) cells which are CD16+CD56+ were significantly increased in HIV positive NHL (HIV+NHL) patients as compared to both HIV negative NHL (NHL) patients (87 ± 47 vs 62 ± 55 ; $p=0.0037$) and HIV positive patients on a cART regimen (HIV+ cART) (87 ± 47 vs 51 ± 39 ; $p=0.0010$) (figure 4.4). As compared to controls, NHL patients had significantly higher numbers of NK-cells (62 ± 55 vs 37 ± 30 ; $p=0.0214$). HIV+ cART patients had significantly lower numbers of NK-cells than cART-naïve HIV+ patients (51 ± 39 vs 81 ± 37 ; $p=0.0018$). cART-naïve HIV+ patients had increased numbers of NK-cells than the controls (81 ± 37 vs 37 ± 30 ; $p=0.0002$).

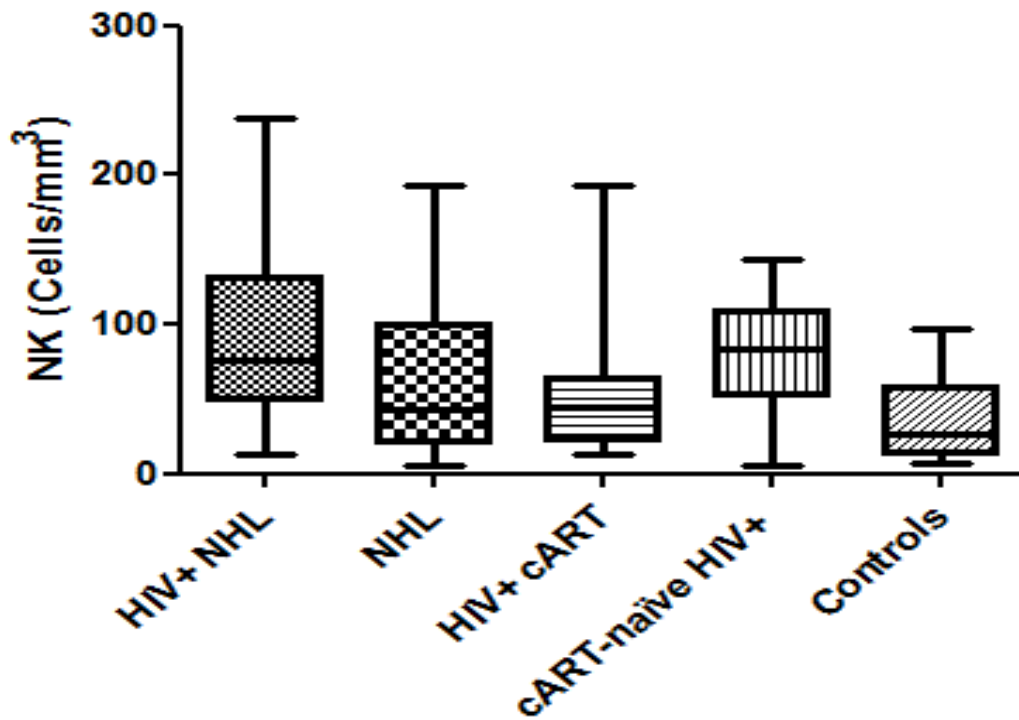


Figure 4.4 *Natural killer (NK) cells.* HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

4.4.4 Natural killer T (NKT) cells

The numbers of natural killer T (NKT) cells were significantly decreased in HIV positive NHL (HIV+NHL) patients as compared to both HIV negative NHL (NHL) patients (161 ± 102 vs 230 ± 177 ; $p=0.0321$) and HIV+ patients on a cART regimen (HIV+ cART) (161 ± 102 vs 238 ± 213 ; $p=0.0361$) (figure 4.5). As compared to the control population, NHL patients had significantly lower numbers of NKT-cells (230 ± 177 vs 334 ± 153 ; $p=0.0243$). There was no significant difference in the numbers of NKT-cells when HIV+ cART patients were compared with cART-naïve HIV+ patients. cART-naïve HIV+ patients had significantly lower numbers of NKT-cells than the controls (217 ± 197 vs 334 ± 153 ; $p=0.0019$).

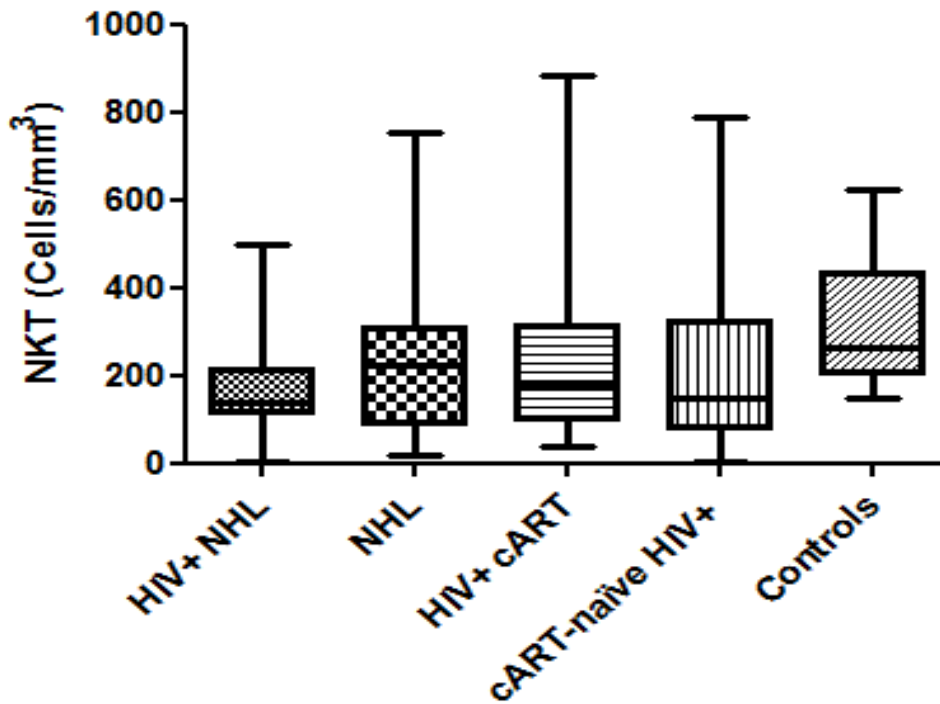


Figure 4.5 *Natural killer T (NKT) cells.* HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

4.5 Discussion

4.5.1 CD4+ T-cells

The primary targets of HIV-1 are CD4 T-cells and it is well established that HIV-1 infection leads to the reduction in CD4 T-cell counts [Alimonti et al., 2003]. CD4 T-cell depletion is one of the hallmarks of progression of HIV-I infection and a major indicator of the stage of the disease in HIV+ patients [Hogg et al., 2001; Fasakin et al., 2014]. In the current study, the numbers of CD4 T-cells were significantly decreased in HIV+NHL patients as compared to NHL (figure 4.1). HIV-1 virus may have decreased the numbers of CD4 T-cells in HIV+NHL patients. In addition, HIV+ cART patients had significantly higher numbers of CD4 T-cells than HIV+NHL patients (figure 4.1). These findings suggest that HIV+NHL patients that are on cART as well as on chemotherapy have lower numbers of CD4 T-cells than HIV+ patients without NHL that are on cART. This may be due to the effect of chemotherapy on CD4 T-cell counts.

It has been previously reported that chemotherapy may be detrimental to the immune system and may lead to decreased CD4 T-cell counts especially in the first few months [Mackall et al., 1994; Proietti et al., 2012; Nars and Kaneno 2013]. Although the immune system improves after few months on chemotherapy, it may not improve to the same extent as it was before. A study conducted by Zanussi and colleagues [1996], showed that mean CD4 T-cell counts declined significantly after the third cycle of chemotherapy in HIV+NHL patients. In addition, NHL patients had significantly lower numbers of CD4 T-cells as compared to the control population in the current study (figure 4.1).

In a study by Bower and colleagues [2009], it has been shown that a lower latest CD4 T-cell count is strongly associated with systemic NHL in HIV+ patients who had not received cART. In the current study, it was observed that HIV+ cART patients had higher numbers of CD4 T-cells than cART-naïve HIV+ patients. Although the nadir CD4 T-cell counts were less than 350 cells/mm³ at the time of recruitment, the CD4 T-cell counts measured in the current study were slightly greater than 350 cells/mm³. HIV+ cART patients were on cART; therefore, their CD4 T-cell counts may have increased as compared to the previous measurements. However, the nadir CD4 T-cell counts at the time of recruitment are not reported in the current study. These results confirm that cART increases the numbers of CD4 T-cells in HIV+ patients while it decreases HIV-1 virus. Wolbers and colleagues [2007], showed that CD4 T-cell count increased with suppressed viral load following cART initiation in HIV-1 infected patients.

Furthermore, in a cohort of HIV+ patients, Smith and colleagues [2003], showed that the CD4 T-cell count increased from 175 cells/mm³ to a median of 319 cell/mm³ following initiation of cART. It has been reported that in HIV+ cART patients who manage to maintain virological suppression, the number of CD4 T-cells continue to increase for at least 3 years after starting cART to the levels seen in HIV negative individuals [Smith et al., 2003]. cART-naïve HIV+ patients had lower numbers of CD4 T-cells than the controls (figure 4.1) in the current study. This confirms that HIV-1 virus reduces the number of CD4 T-cells in infected patients. One of the major mechanisms resulting in CD4 T-cell depletion in cART-naïve HIV+ patients is chronic immune activation that leads to increased T-cell turnover and apoptosis [Massanella

et al., 2013]. However, CD4 T-cell counts may have been lower in cART-naïve HIV+ patients partly due to the inclusion criterion of less than 350 cells/mm³. The reduced CD4 T-cell counts may make HIV+ patients more prone to infections including EBV infection. In a multivariate analysis, Tedeschi and colleagues [2012], showed that low CD4 T-cell count and detectable serum EBV viral load measured at lymphoma diagnoses are independently associated with poor survival among HIV+NHL patients.

4.5.2 CD8 T-cells

HIV-1 infection is characterized by decreasing CD4 T-cell counts and increasing CD8 T-cell counts [Grossman 2003; Catalfamo et al., 2010]. In the current study, there was no significant difference in the numbers of CD8 T-cells between HIV+NHL and NHL patients. Although not significant, there was a trend towards increased numbers in HIV+NHL patients as compared to NHL (figure 4.2). In addition, HIV+NHL patients had significantly higher numbers of CD8 T-cells than HIV+ cART patients (figure 4.2). CD8 T-cells are required for elimination of HIV-1 virus; however, the virus itself is suppressed by cART thus decreasing the number of CD8 T-cells required. The numbers of CD8 T-cells were significantly increased in NHL as compared to controls (figure 4.2). The increased numbers of CD8 T-cells are required to eliminate the HIV-1 virus, EBV and to destroy malignant lymphoma cells in HIV+NHL patients.

However, although the numbers of CD8 T-cells may be increased in NHL and HIV state, their functional activities may be lost. Kostense and colleagues [2002], reported that the activity of HIV specific CD8 T-cells is lost in HIV-1 infected patients, however, this was not due to physical depletion of CD8 T-cells, but is mainly due to impaired function. A study conducted by van Baarle and colleagues [2001], indicated that functional loss of EBV specific CD8 T-cells with concomitant increase in EBV load may play a role in the pathogenesis of HIV+NHL. In the current study, the numbers of CD8 T-cells were significantly reduced in HIV+ cART patients as compared to cART-naïve HIV+ patients (figure 4.2). It has been previously shown that CD8 T-cell expression is mainly driven by HIV-1 RNA levels [Catalfamo et al., 2008].

Due to a reduced HIV-1 viral load in response to cART, only a small number of CD8 T-cells are required in treated HIV+ patients, however, in cART-naïve HIV+ patients, the viral load is high, leading to increased numbers of CD8 T-cells. It has been reported that, during cART mediated viral suppression, HIV+ patients experience increasing CD4 T-cell counts with a simultaneous decline in CD8 T-cell counts [Serrano-Villar et al., 2014]. This was also confirmed when CD8 T-cell counts were compared between cART-naïve HIV+ patients and controls. The numbers of CD8 T-cells were significantly increased in cART-naïve HIV+ patients as compared to the controls (figure 4.2). A study conducted by Ray and colleagues [2006], showed that the numbers of CD8 T-cells were increased in both asymptomatic HIV+ patients and HIV disease patients as compared to HIV negative individuals.

4.5.3 CD19+ B-cells

HIV-1 infection is associated with extensive B-cell abnormalities, manifested by phenotypic alterations and polyclonal B-cell activation, and increased frequencies of B-cell malignancies [Cagigi et al., 2008; Moir et al., 2008]. HIV-1 infection is characterized by depleted B-cell numbers, increased expression of activation and apoptosis markers [Cagigi et al., 2008]. Dysregulated CD19 expression has been associated with abnormalities of the immune system [Mei et al., 2012]. In the current study, no statistical significant difference in the numbers of CD19+ B-cells between HIV+NHL and NHL patients was found (figure 4.3). There was also no significant difference in the numbers of CD19+ B-cells between HIV+NHL and HIV+ cART patients (figure 4.3). However, NHL patients had lower numbers of CD19+ B-cells when compared to the controls (figure 4.3).

HIV+ cART patients had higher numbers of CD19+ B-cells than cART-naïve HIV+ patients. In addition, cART-naïve HIV+ patients had lower numbers of CD19+ B-cells than the controls (figure 4.3). These findings suggest that infection by HIV-1 virus reduces the numbers of CD19+ B-cells and cART increases the number of CD19+ B-cells. In a study of chronically HIV-1 infected patients, Moir and colleagues [2008], reported a reduction in B-cell numbers and the presence of perturbed B-cell subpopulations before cART initiation, however, following one year of cART and reduction in viral load, B-cell numbers partially returned to normal.

4.5.4 Natural killer (NK) cells

NK-cells are deregulated in HIV-1 infection, and its dysfunctions has been associated with the severity of HIV disease and impaired immune responses [Bayigga et al., 2014]. In the current study, the numbers of NK-cells were significantly increased in HIV+NHL as compared to NHL and HIV+ cART patients (figure 4.4). NK-cells are responsible for anticancer surveillance and protection against tumors [Nowicki et al., 2008], hence its expression is increased in HIV+NHL patients in the current study. This is also confirmed by increased numbers of NK-cells in NHL patients as compared to controls (figure 4.4). The numbers of NK-cells were significantly increased in cART-naïve HIV+ patients as compared to HIV+ cART patients and controls (figure 4.4), this corresponds with increased viral load in cART-naïve HIV+ patients.

It has been previously reported that NK-cells are increased in both percentage and absolute number during HIV-1 infection [Mitchai et al., 2014]. However, it has been shown that HIV-1 viremia induces several phenotypic and functional abnormalities in NK-cells [Mitchai et al., 2014]. Naranbhai and colleagues [2013], reported an increase in NK-cell activation but reduced NK-cell cytotoxicity during acute HIV-1 infection. Thus, although the numbers of NK-cells may be increased in this patient population they may be dysfunctional. It has also been demonstrated that NK-cells are targets for HIV-1 infection in both in vitro and in vivo, and HIV-1 infection of NK-cells is important for virus persistence [Valentin et al., 2002]. Furthermore, HIV-1 has been reported to affect the ability of NK-cells to secrete cytokines and chemokines, which are essential for an effective NK-cell response [Funke et al., 2011]. Decreased

function and number of NK-cells have been associated with rapid HIV disease progression [Nowicki et al., 2008].

4.5.5 Natural killer T (NKT) cells

Previous studies indicate that NKT-cells can display cytotoxic activity against several tumor cell lines such as haemopoietic malignancies, but they show a killing pattern distinct from conventional T and NK-cells [Tarazona et al., 2003; Wu and Van Kaer 2011]. However, a subset of NKT-cells express the CD4 T-cell receptor on their surface, therefore, they may be vulnerable to direct infection by HIV-1 [Unutmaz 2003; Vasan and Tsuji 2010]. In the current study, the numbers of NKT-cells were significantly reduced in HIV+NHL as compared to both NHL and HIV+ cART patients (figure 4.5). The reduced numbers of NKT-cells observed in HIV+NHL in the current study may have been caused by HIV-1 infection.

It has been reported that NKT-cells are rapidly depleted in HIV-1 infected patients and the depletion appears to be due to direct infection of CD4 expressing NKT-cells [Li and Xu 2008; Rout et al., 2012]. The numbers of NKT-cells were significantly reduced in NHL as compared to controls. No significant difference was observed between HIV+ cART and cART-naïve HIV+, however there was a trend towards decreased NKT-cells in cART-naïve HIV+. In addition, cART-naïve HIV+ patients had lower numbers of NKT-cells than the controls. These results are consistent with previous studies. In a cross-sectional study of chronically infected HIV+ patients, Van der Vliet and colleagues [2002], showed that circulating numbers of NKT-cells are

reduced in HIV+ state regardless of CD4+ T-cell counts, CD4:CD8 ratios, HIV plasma viral load and irrespective of cART treatment. Furthermore, Vasan and colleagues [2007], in a cohort of acute HIV-1 infected patients, showed that the patients that initiated cART did not experience a rise in percentage of circulating NKT-cells after 1 year of cART as compared to pre-treatment levels. However, they also did not experience any decline in NKT-cell levels, suggesting that cART may be playing a role in stabilizing the rate of NKT-cell depletion by HIV-1 [Vasan et al., 2007].

4.6 Conclusion

HIV+NHL patients have suppressed CD4 T-cell count, and NKT-cells and increased numbers of NK-cells, and CD8 T-cells. There was no significant difference in the numbers of CD19+ B-cells. These results imply that the immune system of HIV+NHL is impaired and this may be due to the effect of HIV-1 infection on these cells. cART increased the numbers of CD4 T-cells and NKT-cells while it reduced the numbers of CD8 T-cells and NK-cells in HIV+ patients.

CHAPTER FIVE

T-CELL ACTIVATION AND REGULATORY MARKERS

5.1.1 Introduction

Chronic immune activation is hallmarked by an overtly activated immune system, which includes aberrant activation of the adaptive immune system comprising T and B-cells [Hass et al., 2011]. HIV-1 infection is characterized by CD4 T-cell depletion, CD8 T-cell expansion, and chronic immune activation that lead to immune dysfunction [Catalfamo et al., 2011]. In addition, increased immune activation in patients on long term suppressive cART has been associated with increased mortality and both AIDS and non-AIDS defining illnesses [Rajasuriar et al., 2013]. This suggests that chronic immune activation may have a potential role in driving increased morbidity and mortality.

The mechanisms involved in systemic immune activation in chronic HIV-1 infection are multifactorial and include the translocation of microbial products from the gastrointestinal tract, low level HIV viremia, and co-infections with other persistent viral pathogens [Rajasuriar et al., 2013]. The excessive production of interferon alpha (IFN- α) and pro-inflammatory cytokines leading to up-regulation of pro-apoptotic molecules, lymph node fibrosis, and dysfunction of regulatory T-cells may also likely contribute [Rajasuriar et al., 2013].

5.1.2 T-cell activation

Previous studies have shown that HIV-1 infected individuals have elevated levels of immune activation during untreated disease and that these levels do not normalize even with long term treatment with cART [Hatano 2013]. HIV-1 infection is associated with a state of excessive T-cell activation, which has been shown to be a strong prognostic indicator for disease progression at different stages of HIV-1 infection [Cao et al., 2009]. T-cell activation during HIV-1 infection is closely linked to CD4+ T-cell depletion and viral replication [Haas et al., 2011]. This aberrant activation of T-cells is observed mainly for memory CD4+ and CD8+ T-cells and is documented by increased expression of surface activation markers CD38 and human leukocyte antigen-DR (HLA-DR) [Cohen Stuart et al., 2000; Haas et al., 2011].

It has been reported that the majority of these activated T-cells are neither HIV specific nor HIV-1 infected [Douek et al., 2002; Haas et al., 2011]. Lymphocyte hyper-activation and increased serum concentrations of inflammatory cytokines are observed in both treated and untreated HIV-1 infected patients [Ouedraogo et al., 2013]. Chronic immune activation during HIV-1 infection leads to increased T-cell turnover/exhaustion and lymph node fibrosis [Ring 2011]. The increased concentrations of both soluble biomarkers of inflammation and markers of T-cell activation have been shown to be associated with and predictive of increased morbidity and mortality in treated HIV-1 infection [Hatano 2013].

5.1.3 T-cell regulation

The T-cell compartment of the immune system is composed of a large repertoire of T-cell clones, each equipped with a unique antigen receptor, and thus, they can react with an enormous variety of antigens including self-antigens [Romagnani 2006]. The balance between immune activation required for optimal host defence against infection and immune suppression that maintains self-tolerance by preventing autoimmunity is stringently regulated [Rowe et al., 2012]. Regulatory T (T-reg) cells play a role in maintaining immune homeostasis, preventing autoimmunity, moderating inflammation, and minimizing collateral tissue damage [Feuerer et al., 2009; Bilate and Lafaille 2012]. They maintain tolerance to self and control autoimmune deviation to prevent uncontrolled responses to pathogens or allergens, help maintain a balance with obligate microbial flora, and facilitate tumor escape from immune monitoring [Feuerer et al., 2009].

The primary function of T-reg cells is to inhibit the function of antigen presenting cells and effector cells. T-regs can be generated when developing T-cells encounter TCR agonist ligands in the thymus [Kretschmer et al., 2008]. Two main types of T-regs have been identified, i.e. natural and induced (or adaptive) and they both play a role in turning down effector immune responses [Bilate and Lafaille 2012]. Inducible subset of T-regs are type 1 regulatory (Tr1) cells and they exert their function by secreting high concentrations of IL-10 and the killing of myeloid cells through the release of granzyme B [Gregori et al., 2012]. Naturally occurring T-regs are CD4⁺CD25⁺ and they specifically express the transcription factor FoxP3 (Forkhead box protein 3) which is essential for their development and function [Thompson and

Powrie 2004; Romagnani 2006; Sakaguchi et al., 2008]. Reduced numbers or function of T-reg cells have been associated with the onset of autoimmunity, whereas increasing their numbers has had therapeutic success in models of autoimmunity and graft-versus-host disease [Bailey-Bucktrout et al., 2013]. In addition, the reduced numbers of T-reg cells may result in increased T-cell activation without monitoring.

5.2 Specific Aims

The aim of this study was to determine,

- The expression of CD38 on CD8+ T-cells in HIV+NHL patients and control populations
- The expression of FoxP3 in HIV+NHL patients and control populations
- Whether cART increases FoxP3 expression and down-regulates T-cell activation

5.3 Materials and Methods

5.3.1 Materials

The following antibodies, materials and reagents were purchased from the Scientific Group Pty Ltd supplier of Becton Dickinson antibodies: HU CD38-PE MAB (Catalogue number: BD/560381), HU CD8-FITC MAB (Catalogue number: BD/560960), APC labeled anti-human CD4 (Catalogue number: BD/555349), CD45-FITC MAB (Catalogue number: BD/345808), CD3-PerCP MAB (Catalogue number: BD/345766), FoxP3-PE MAB (Catalogue number: BD/560082), FoxP3 buffer set (Catalogue number: BD/560098), Phosphate buffered saline (PBS) (Catalogue number: P5368-10PAK), and FACS Lysing Solution (Catalogue number: BD/349202), Ethylenediaminetetraacetic acid (EDTA) tube (Catalogue number: BD367864) and BD falcon tubes (Catalogue number: BD/352054). Molecules of Equivalent Soluble Fluorochrome (MESF) beads (Catalogue number: 827B/10220) were purchased from Bangs laboratory.

5.3.2 Methods

5.3.2.1 Sample preparation

The samples were collected and prepared according to the methods described in section 4.3.2.1 and 4.3.2.2.

5.3.2.2 Protocol

5.3.2.2.1 CD8+CD38 Expression

CD38 expression on CD8 T-cells (CD8+CD38) was determined as follows. BD Falcon tubes were labelled for each sample and beads. Twenty µl of the following monoclonal antibodies were added into all tubes, CD3 PerCP, CD8 FITC, and CD38 PE. Fifty µl of each sample was added into tube containing antibodies. The tubes were vortexed gently and the samples were incubated for 15 minutes in the dark cupboard at room temperature. Four hundred and fifty µl of FACS lysing solution was added into all tubes and samples were incubated again for 15 minutes at room temperature in the dark cupboard. Beads were prepared as follows, 2 falcon tubes were labelled one for mixed beads and one for the blank. Four hundred and fifty µl of FACS lysing solution was added into both tubes. Blank beads were added into both tubes and the mixed beads were added into the second tube in addition to the blank. Following 15 minutes incubation, all samples were analysed immediately, starting with the prepared beads.

5.3.2.2.2 FoxP3 Expression

FoxP3 expression was determined as follows. BD Falcon tubes were used during sample analysis. Twenty μl of CD45-FITC, CD3-PerCP, and CD4-APC were added to falcon tubes which were labelled according to each sample to be analysed and standards of lymphocyte subsets (lymphosures) low and normal [Synexa Life Sciences 2009]. Hundred μl of sample and lymphosures were added to the tubes containing antibodies. Tubes were vortexed and samples were incubated for 20 minutes in the dark cupboard at room temperature (20-25°C). Following the incubation, 900 μl of FACS lysing solution was added into all tubes, vortexed and samples were incubated for 15 minutes at room temperature in the dark cupboard. Following the incubation, samples were centrifuged for 5 minutes at 2000 rpm.

The supernatant was decanted and the resultant pellet was re-suspended in the residual volume of FACS lyse by vortexing gently. Two ml of phosphate buffered saline (PBS) was added to all samples and samples were centrifuged at 2000rpm for 5 minutes. The supernatant was decanted and the resultant pellet was re-suspended in the residual volume of PBS. Five hundred μl of Buffer C (*Appendix III*) was added to all samples, tubes vortexed and were incubated for 30 minutes at room temperature in the dark cupboard. Following 30 minutes of incubation, samples were then centrifuged, supernatant decanted and the resultant pellet was re-suspended in the residual volume of buffer C. Twenty μl of FoxP3 PE antibody was added into all samples, vortexed and the samples were incubated for 30 minutes at room temperature in the dark cupboard. Following the incubation period, 2ml of PBS was added into all samples, and were centrifuged at 2000rpm for 5 minutes.

The supernatant was decanted and the resultant pellet was re-suspended in the residual volume of PBS. Fifty μl of 5% fixative was then added into all samples and the samples were analysed within 24 hours.

5.4 Results

5.4.1 CD8+CD38 Expression

CD8+CD38 expression was significantly up-regulated in HIV positive NHL (HIV+NHL) patients as compared to HIV positive patients on a cART regimen (HIV+cART) (10.8 ± 7.80 vs 7.36 ± 6.90 ; $p=0.0104$), however, there was no significant difference between HIV+NHL and NHL (figure 5.1). NHL patients had higher CD8+CD38 expression than controls (9.56 ± 5.53 vs 3.65 ± 1.48 ; $p<0.0001$). HIV+cART patients had significantly lower CD8+CD38 expression than cART-naïve HIV+ patients (7.36 ± 6.90 vs 15.95 ± 8.81 ; $p<0.0001$). cART-naïve HIV+ patients had higher CD8+CD38 expression than controls (15.95 ± 8.81 vs 3.65 ± 1.48 ; $p<0.0001$).

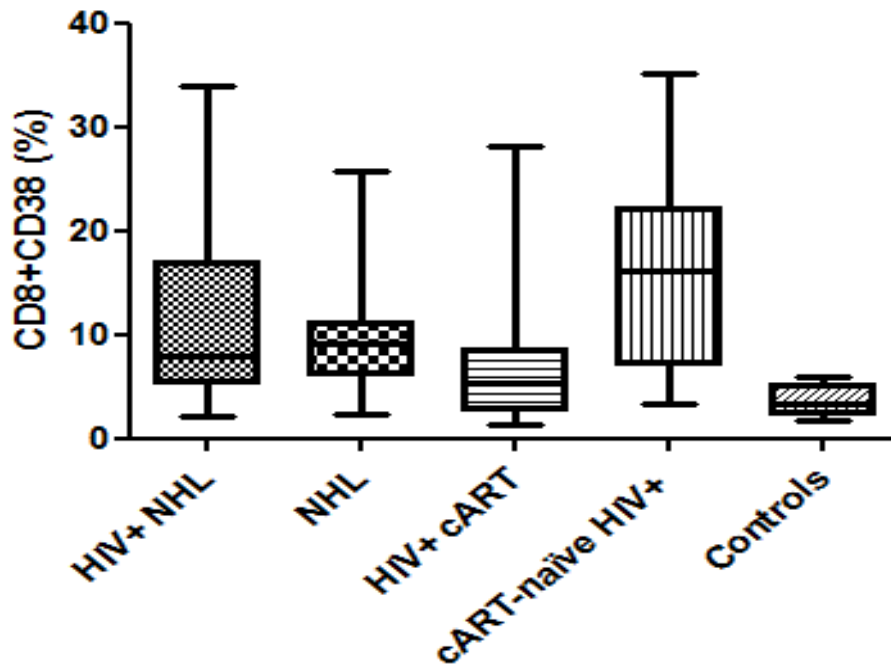


Figure 5.1 *CD8+CD38 expression.* HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

5.4.1.1 CD8+CD38 Correlations

The expression of CD8+CD38 was negatively associated with the numbers of CD4 T-cells ($r=-0.4345$, $p<0.0001$); CD19+ B-cells ($r=-0.4814$, $p<0.0001$); NKT-cells ($r=-0.2132$, $p=0.0056$); and FoxP3 expression ($r=-0.2033$, $p=0.0078$) (figure 5.2A-D). CD8+CD38 expression decreased significantly with increasing numbers of CD4 T-cells, CD19+ B-cells, as well as increasing FoxP3 expression. Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association was observed between CD8+CD38 and HIV+NHL.

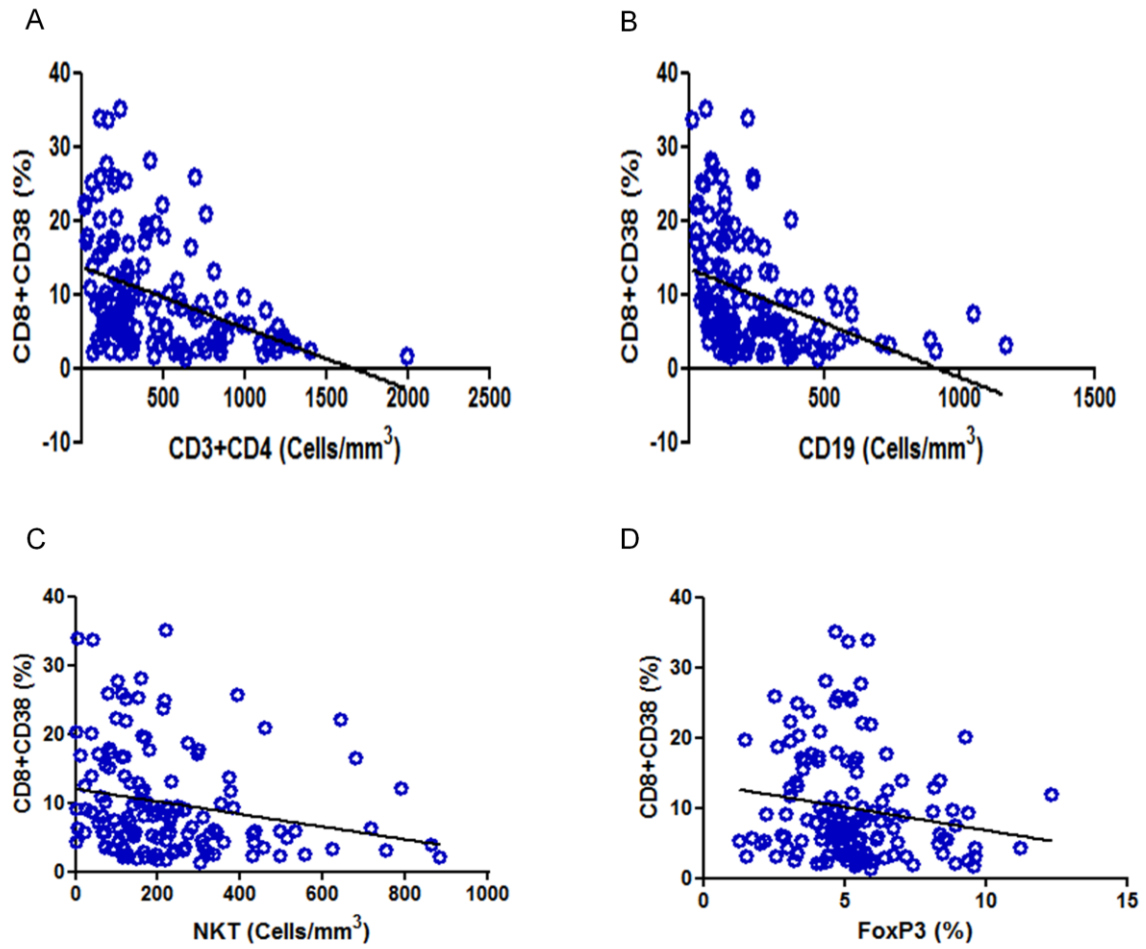


Figure 5.2 *CD8+CD38 Correlations.* A) CD4 vs CD8+CD38 B) CD19 vs CD8+CD38 C) NKT cells vs CD8+CD38 D) FoxP3 vs CD8+CD38. NKT, Natural killer T-cells; FoxP3, Forkhead box protein 3.

5.4.2 FoxP3 Expression

The expression of FoxP3 was significantly down-regulated in HIV positive NHL (HIV+ NHL) patients as compared to both HIV negative NHL (NHL) patients (4.28 ± 1.87 vs 6.37 ± 2.04 ; $p < 0.0001$) and HIV positive patients on a cART regimen (HIV+ cART) (4.28 ± 1.87 vs 5.02 ± 0.91 ; $p = 0.0171$) (figure 5.3). NHL patients had significantly lower FoxP3 expression than controls (6.37 ± 2.04 vs 7.59 ± 1.70 ; $p = 0.0251$). As compared to cART-naïve HIV+ patients, HIV+ cART patients had significantly higher FoxP3 expression (5.02 ± 0.91 vs 4.02 ± 1.28 ; $p = 0.0059$). In addition, cART-naïve HIV+ patients had significantly lower FoxP3 expression than controls (4.02 ± 1.28 vs 7.59 ± 1.70 ; $p < 0.0001$).

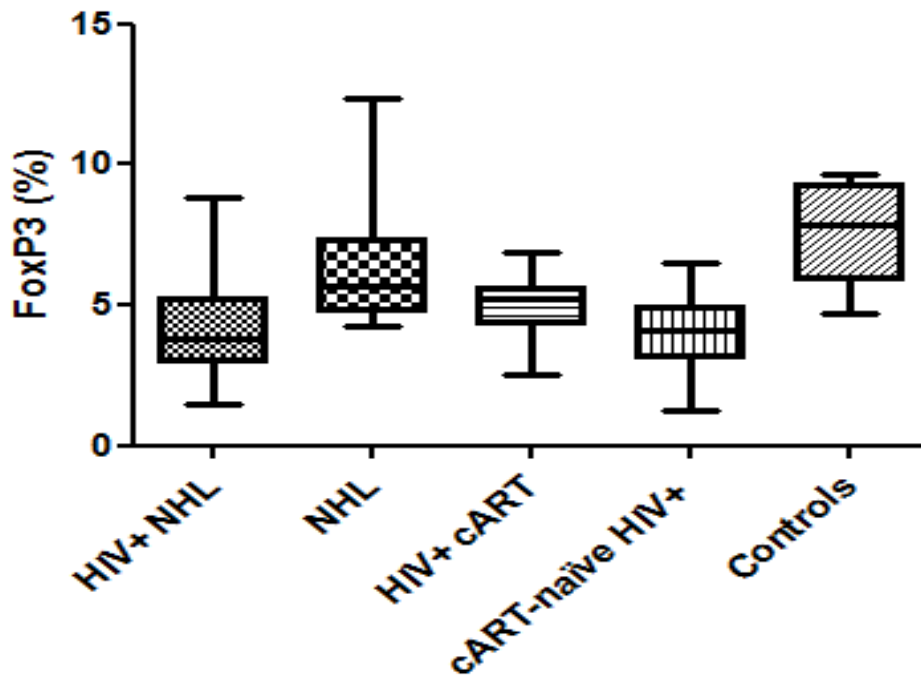


Figure 5.3 *FoxP3* expression. HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

5.4.2.1 FoxP3 Correlations

The expression of FoxP3 was positively correlated with the numbers of CD4 T-cells ($r=0.2979$, $p=0.0002$), and CD19+ B-cells ($r=0.2465$, $p=0.0016$) (figure 5.4A and C), while it was negatively associated with the numbers of CD8 T-cells ($r=-0.2701$, $p=0.0006$) and NK-cells ($r=-0.2010$, $p=0.0084$) (figure 5.4B and D). In addition, as mentioned previously FoxP3 was negatively associated with CD8+CD38 expression (section 5.4.1.1). Following adjustment for age, gender, smoking status, viral load and duration of treatment, the serum concentrations of FoxP3 were independently associated with HIV+NHL (OR = 0.68; 95% CI = 0.44–1.04).

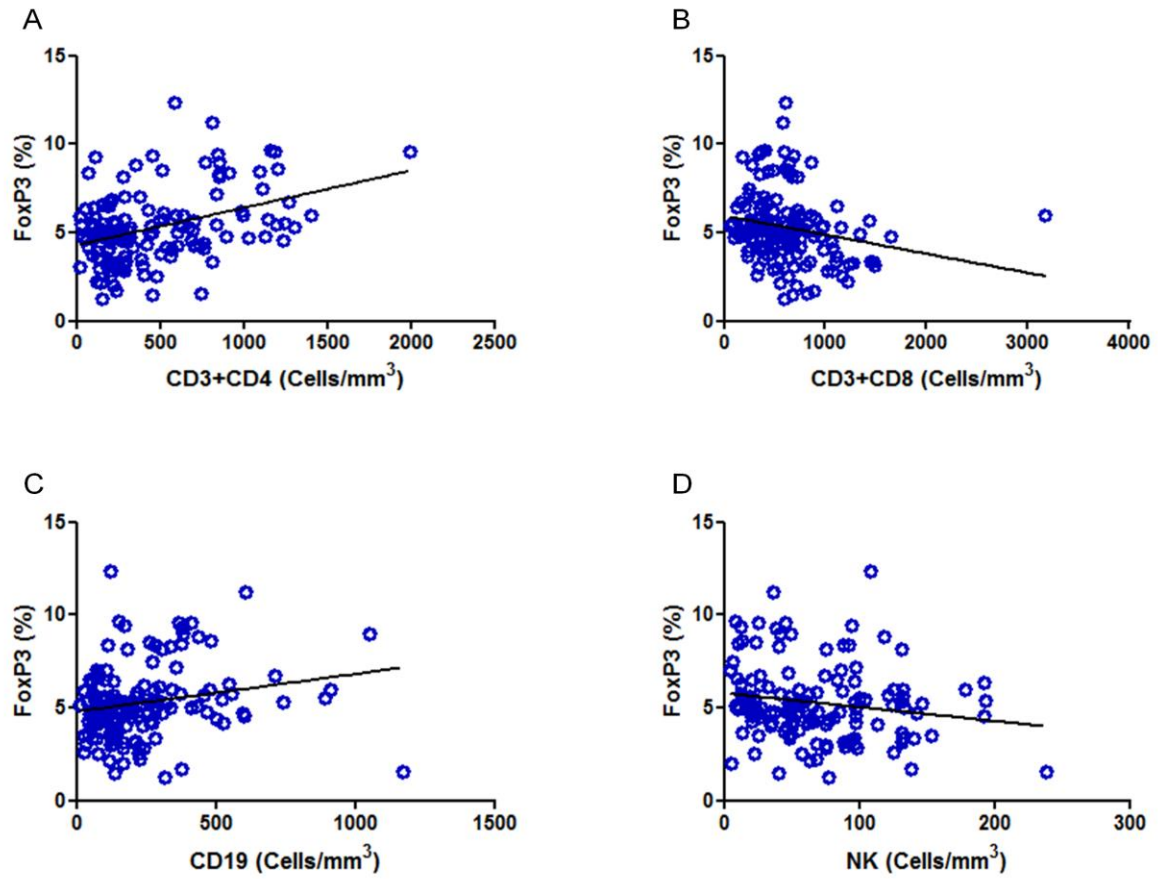


Figure 5.4 *FoxP3* Correlations. A) CD4 vs *FoxP3*; B) CD8 vs *FoxP3*; C) CD19 vs *FoxP3* D) NK vs *FoxP3*. *FoxP3*, Fork head box protein 3; NK, Natural killer cells

5.5 Discussion

5.5.1 CD8+CD38 Expression

Chronic immune activation has been suggested to be one of the mechanisms leading to the development of NHL in HIV+ patients [Epeldegui et al., 2010]. Increased expression of CD38 on CD8 T-cells (CD8+CD38) has been previously associated with immune activation, progression of HIV disease, and death [Sherman et al., 2002]. In the current study, T-cell activation in HIV+NHL patients was investigated. There was no significant difference in the expression of CD8+CD38 between HIV+NHL and NHL patients. However, although there was no significant difference, there was a trend towards increased CD8+CD38 expression in HIV+NHL patients. In addition, HIV+NHL had significantly elevated T-cell activation as compared to HIV+ cART patients (figure 5.1).

CD8+CD38 has been previously shown to function as a signalling molecule in B-cell chronic lymphocytic leukemia (B-CLL) and has been linked with disease pathogenesis [Deaglio et al., 2006; Tinhofer et al., 2006]. CD8+CD38 expression has been shown to be an important prognostic marker in B-CLL that is stable over time and is not significantly influenced by chemotherapy [Dürig et al., 2002]. Furthermore, as compared to the controls, NHL patients had increased T-cell activation (figure 5.1). The increased T-cell activation observed in NHL patients may have been caused by EBV infection or anti-tumor immune response. CD8 T-cell activation may be necessary for killing malignant lymphoma cells in NHL. To confirm if HIV-1 infection leads to increased T-cell activation, the levels of T-cell activation between

cART-naïve HIV and control individuals were compared. T-cell activation was significantly elevated in cART-naïve HIV+ patients as compared to the controls. Benito and Colleagues [2004], showed that the levels of CD8+CD38 are increased in untreated HIV-1 infection, and are strongly associated with plasma viremia. Following cART initiation, CD8+CD38 levels declined steadily in HIV+ patients [Benito et al., 2004]. Furthermore, Resino and colleagues [2004], showed that HIV-1 infected children with high CD8+CD38 levels had a higher incidence and relative risk of virological failure than those with lower CD8+CD38. In addition, the effect of cART in T-cell activation was studied. T-cell activation was significantly up-regulated in cART-naïve HIV+ patients as compared to HIV+ cART patients (figure 5.1). These results suggest that HIV-1 increases T-cell activation and the cART initiation decreases T-cell activation.

Consistent with the current findings, Deeks and colleagues [2004], reported that the initiation of cART during early HIV-1 infection reduces the level of CD8 T-cell activation. In addition, Almeida and colleagues [2007], showed that prior to cART, CD38 expression was increased on peripheral blood CD8 T-cells, cART initiation decreased CD38 expression significantly, however, its level of expression remained abnormally high after one year of therapy. These findings confirm that CD8+CD38 T-cell count is a good prognostic marker in HIV-1 infected patients and can predict treatment response [Resino et al., 2004; Coetzee et al., 2009; Rönsholt et al., 2012]. In the current study, the elevated T-cell activation was negatively associated with the numbers of CD4 T-cells, CD19+ B-cells, NKT-cells, and FoxP3 expression (figure 5.2). These results suggest that as the T-cell activation increases, CD4 T-cell count

decreases in HIV+ patients. In addition, T-cell activation increased with decreasing FoxP3 expression (figure 5.2). These results suggest that the increased T-cell activation observed in HIV+ patients is associated with decreased T-cell regulation. The expression of FoxP3 which normally inhibit T-cell activation is reduced, thus T-cell activation occurs continuously without regulation. This in turn may lead to chronic immune activation which may result in increased CD8 and CD4 T-cell turnover and rapid progression of HIV disease.

5.5.2 FoxP3 Expression

FoxP3 plays an important role in regulatory T-cell (T-reg) function, development and maintenance [Holmes 2008]. T-reg cells have been implicated in the suppression of T-cell activation, proliferation and cytokine production [Card et al., 2009; Presicce et al., 2011]. Dysregulated T-reg cell expression has been associated with a number of pathological conditions including cancer, infectious and autoimmune diseases [Holmes et al., 2008a]. In the current study, the expression of FoxP3 in HIV+NHL patients was investigated. The expression of FoxP3 was significantly down-regulated in HIV+NHL as compared to NHL as well as when compared to HIV+ cART patients (figure 5.3). The reduced FoxP3 expression observed in HIV+NHL may have been caused by HIV-1 infection, as the FoxP3 levels are higher in HIV negative NHL and cART treated HIV+ patients. This may have detrimental effects on T-cell regulation and activation.

FoxP3 expression was also down-regulated in NHL patients as compared to the controls (figure 5.3). This may have been caused by infection by EBV or the detrimental effect of CHOP in the immune system. In a study conducted by El-Sayed and colleagues [2013], it was shown that mRNA transcripts as well as percentages of FoxP3 were significantly increased in B-cell NHL patients before receiving CHOP, when compared to healthy controls, however, after 6 cycles of CHOP treatment FoxP3 expression decreased significantly. These results suggest that T-cell regulation is impaired in both NHL and HIV+ state.

As mentioned previously, one of the hallmark features of NHL is chronic immune activation, which may be due to suppressed T-cell regulation. In addition, the reduced T-reg cell expression observed in HIV negative NHL patients may be beneficial as they may lead to increased immune activation and anti-tumoral responses, while the increased T-reg cell expression could limit the anti-tumor immune response, favouring tumor growth and development [El-Sayed et al., 2013; Simonetta and Bourgeois 2013]. To investigate the effect of cART on T-cell regulation, FoxP3 expression between HIV+ cART and cART-naïve HIV+ patients were compared. The expression of FoxP3 was significantly increased in HIV+ cART than cART-naïve HIV+ patients (figure 5.3). Thus cART may have increased the expression of FoxP3 in this population group. Consistent with the current findings, Andersson and colleagues [2005], reported suppressed FoxP3 expression in cART-naïve HIV+ patients, however, upon initiation of cART, the levels of FoxP3 expression normalized. cART-naïve HIV+ patients also had decreased FoxP3 expression as compared to the controls (figure 5.3).

This confirms that HIV-1 infection decreases T-cell regulation leading to chronic T-cell activation and turn-over. T-reg cells have been shown to be susceptible to HIV-1 infection [Moreno-Fernandez et al., 2009]. These findings are consistent with previous studies. It has been previously shown that FoxP3 mRNA levels are decreased in peripheral blood CD4 T-cells from HIV+ patients as compared to uninfected individuals [Apoil et al., 2005; Simonetta et al., 2013]. In addition, the expression of FoxP3 was positively associated with the numbers of CD4 T-cells, and CD19+ B-cells (figure 5.4).

The reduced FoxP3 expression observed in the current study, may reflect the impact of HIV-1 on CD4 T-cells [Simonetta et al., 2013]. As these patients recover, as a result of cART use, their T-cell regulation increases. FoxP3 expression was negatively associated with CD8+CD38, CD8, and NK-cells (figure 5.4), that are increased in HIV-1 infection. In addition, FoxP3 expression was independently associated with HIV+NHL. These findings suggest that FoxP3 expression decreases with decreasing CD4 T-cell count in HIV+ state, resulting in suppressed T-cell regulation. The depleted T-cell regulation that normally inhibit T-cell activation, may lead to uncontrolled persistent and chronic immune activation which in turn may result in rapid HIV disease progression.

5.6 Conclusion

T-cell activation is increased in NHL, as evidenced by increased CD8+CD38 expression in HIV+NHL compared to treated HIV+ patients as well as in NHL as compared to controls. The influence of HIV-1 infection on T-cell activation in HIV+NHL was not clearly defined in the current study, as there was no significant difference between HIV+NHL and HIV negative NHL. However, the current findings confirm that T-cell activation is greatly increased in untreated HIV-1 infection. The observed chronic T-cell activation in HIV-1 infected patients may have been caused by a decreased regulatory T-cell expression. This may lead to increased T-cell turnover and exhaustion resulting in immune dysfunction. cART decreases T-cell activation while increasing its regulation. This data provides additional support for the recommendation that early cART initiation could be beneficial.

CHAPTER SIX

B-CELL ACTIVATION MARKERS

6.1 Introduction

It is well established that prolonged HIV-1 infection causes immune dysfunction including chronic immune suppression and B-cell hyperactivation [Hussain et al., 2013]. Systemic chronic immune activation has been shown to be the primary driving force in HIV pathogenesis [Miedema et al., 2013; Paiardini and Muller-Trutwin 2013]. The causes of HIV associated immune activation are multifactorial and include the translocation of microbial products from the gastro-intestinal tract, low level HIV viremia and co-infection with other persistent viral pathogens [Taiwo et al., 2013; Rajasuriar et al., 2013].

There are two major avenues for B-cell activation, i.e. the activation that occurs in the context of cognate interaction with activated T-cells, whose receptor recognizes antigen presented by the B-cells, or activation by T-cell independent antigens [Bishop et al., 2003]. The mechanisms underlying B-cell hyper-activation in HIV-1 infected individuals is poorly understood, however, it has been characterized by elevated expression of activation/co-stimulatory markers, spontaneous cytokine expression, hyper-gammaglobulinemia, and B-cell malignancies [Siewe et al., 2013]. B-cell activation is also characterized by lymphocyte proliferation, class switch recombination, and somatic hypermutation, all of which are prone to resultant errors in DNA that may lead to lymphomagenesis [De Roos et al., 2012]. Several studies indicate that those patients with the most marked B-cell activation are at increased

risk of developing NHL [Grulich et al., 2000; Purdue et al., 2009; De Roos et al., 2012]. The risk of developing NHL of the B-cell type is greatly increased in HIV+ patients, particularly DLBCL and BL [Breen et al., 2011; De Roos et al., 2012]. Chronic immune activation and B-cell hyper-stimulation may contribute to the risk of NHL development among HIV-1 infected individuals [Marks et al., 2013]. Chronic immune activation is characterized by aberrant cytokine production, perturbation in lymphocyte subsets, and increased lymphocyte turnover is a critical hallmark of HIV-1 infection and disease progression [Siewe et al., 2013]. We hypothesize that biomarkers of immune activation including soluble CD23 (sCD23), sCD27, sCD30 and sCD44 are elevated in HIV+NHL.

6.1.1 Soluble CD20 (sCD20)

CD20 is a 33 kDa B-cell specific antigen that is expressed from the early pre-B-cell stage of development and is lost on differentiation into plasma cells [Roberts et al., 2002]. CD20 antigen is a membrane bound protein that is thought to play a role in B-cell activation, differentiation, and cell cycle progression [Johnson et al., 2009; Li et al., 2012]. It is an excellent pan B-cell immunophenotypic marker as CD20 is highly expressed on the surface of 90-95% of normal and neoplastic B lymphocytes [Li et al., 2012]. CD20 is not expressed on immature B precursors and plasma cells [Li et al., 2012]. It has a 44 amino acid extracellular domain that is a potential target for immunotherapy [Roberts et al., 2002].

In addition, monoclonal antibodies to CD20 such as rituximab have proven to be an effective immunotherapy for B-cell lymphomas [Roberts et al., 2002; Cang et al., 2012]. Therefore the study of CD20 expression in lymphoma cells is vital not only to establish an accurate diagnosis but also to prepare an appropriate plan of treatment with biological drugs [Prevodnik et al., 2011]. It has been confirmed that in the majority of B-cell lymphomas, the CD20 antigen is expressed on the surface of neoplastic cells, however, the intensity of CD20 expression varies by the type of lymphoma and by the differentiation of lymphoma B-cells [Prevodnik et al., 2011].

6.1.2 Soluble CD23 (sCD23)

CD23 is a 45 kDa transmembrane protein identified as the low affinity receptor of IgE and is an adhesion molecule expressed in activated mature B-cells [Fournier et al., 1992; Lopez-Matas et al., 2000]. The soluble form of CD23 (sCD23) is produced by the cleavage of cell surface CD23 and may be involved in the regulation of IgE production [Yawetz et al., 1995]. sCD23 products have a variety of functional activities such as survival extension of B-cells and the induction of cell growth and differentiation not only of B-cells but also of myeloid and T-cells [Lopez-Matas et al., 2000]. It is a marker present in mediastinal B-cells [Salama et al., 2010].

It has been shown that IL-4, and IL-2 are the strong inducers of CD23 expression on most cell types including normal B-cells and monocytes while it may be up or down-regulated by interferon- γ (IFN- γ) depending upon the cell type [Fournier et al., 1992]. Elevated concentrations of sCD23 were found in the serum of patients with several disease states associated with elevated IgE or with enhanced B-cell activation and humoral immunity [Yawetz et al., 1995]. sCD23 concentrations are strongly associated with disease progression and shorter survival in patients with B-cell chronic lymphocytic leukemia [Lesesve et al., 2001].

6.1.3 Soluble CD27 (sCD27)

CD27 is a glycosylated, type I transmembrane protein of about 55 kDa and exists as a homodimer with a disulphide bridge linking the two monomers [Prasad et al., 1997]. It is a member of the tumor necrosis factor receptor (TNF-R) superfamily and is expressed on the surface of T, B, and NK-cells [Prasad et al., 1997; Akiba et al., 1998; Vitale et al., 2012; Song et al., 2012]. These receptors are known to play an important role in cell growth and differentiation, as well as apoptosis or programmed cell death [Prasad et al., 1997]. CD27 signalling activates NF- κ B, promotes cell survival, enhances antigen receptor mediated proliferative signals, and increases effector function [Reither et al., 2012; Claus et al., 2012]. However, CD27 contribution to the immune response is dependent upon CD70 expression, which is primarily controlled by antigen receptor and Toll like receptor stimulation [Borst et al., 2005].

The interaction of CD27 with CD70 plays an important role in the activation, proliferation, and survival of T-cells; in clonal B-cell expansion and germinal centre formation; and in NK-cell cytolytic activity [Vitale et al., 2012; Reither et al., 2012]. CD27 is widely used as a leukocyte differentiation marker for subset T, B and NK-cells and is also recognised as a marker for memory B-cells and is held to be of diagnostic/predictive value in common variable immunodeficiency [van Montfrans et al., 2012]. It has been reported that CD27 deficiency in humans is a new molecularly defined primary immunodeficiency disease associated with persistent symptomatic EBV viremia, hypogammaglobulinemia and impairment in specific antibody function resulting from disturbed CD8⁺ T-cells and T-cell dependent B-cell responses [van

Montfrans et al., 2012]. In addition, it has been shown that CD4⁺ T-cell help and CD27 stimulation supports CD8⁺ T-cell memory by modulating the expression of cytokine receptors that influence the differentiation and survival of memory CD8⁺ T-cells [Dong et al., 2012]. Soluble CD27 is a 32 kDa protein that is identical to the membrane bound CD27 [Huang et al., 2013].

It can be released after lymphocyte activation by differential splicing of the receptor protein or shedding from the cell surface by metalloproteinases [Huang et al., 2013]. Unlike CD27, sCD27 has been detected in serum, plasma, and urine samples from healthy individuals, and increased concentrations have been documented in systemic lupus erythematosus, viral infections, and lymphoid malignancies [Huang et al., 2013]. The concentrations of sCD27 in plasma samples have been used as a marker of disease burden in Waldenstroms macroglobulinemia and to monitor immune activation during antiretroviral therapy in HIV-1 infected patients [De Milito et al., 2002; Huang et al., 2013].

6.1.4 Soluble CD30 (sCD30)

CD30 is a type I transmembrane glycosylated protein of 120/105 kDa derived from 90 kDa non-glycosylated precursor [Horie and Watanabe 1998; Blazar et al., 2004; Albrecht et al., 2014]. It is a transmembrane glycoprotein and a member of the TNF-R superfamily [Gardner et al., 2001; Sotomayor et al., 2014]. CD30 is expressed in a subset of activated B, T lymphocytes and NK-cells but not in resting mature B or T-cells and is rarely expressed on non-neoplastic cells outside the immune system [Vega 2013]. The role of CD30 in lymphocyte biology is not completely understood, but it seems that CD30 signaling participates in the generation and maintenance of both memory B and T-cells, proliferation of B-cells and enhancement of immunoglobulin production [Vega 2013].

CD30 activation can lead to a series of pleiotropic effects resulting in proliferation, differentiation, or survival, depending upon the cell type, activation state and transformation status, as well as the particular signalling pathway that is triggered [Sotomayor et al., 2014]. CD30 expression exhibits limited expression in health, being predominantly expressed on activated T and B-cells [Buchan and Al-Shamkhani 2012]. In cancer CD30 is most consistently expressed by Reed-Sternberg cells of Hodgkin lymphoma and a group of neoplasms known as anaplastic large cell lymphoma [Buchan and Al-Shamkhani 2012]. It is expressed on many lymphomas of B, T, and NK-cell origin [Podack et al., 2002]. Soluble CD30 (sCD30) is about 85 kDa and is cleaved from the surface of CD30+ cells by the cell surface metalloproteinase TNF- α converting enzyme [Schlaf et al., 2007].

Increased serum concentrations of sCD30 are seen in patients with CD30 positive neoplasms, some viral infections, and T_H2 type immune response [Horie and Watanabe 1998]. It has been shown that low serum concentrations of sCD30 are found in healthy individuals, whereas increased sCD30 serum concentrations are detected under pathophysiological situations [Schlaf et al., 2007].

6.1.5 Soluble CD44 (sCD44)

CD44 is a transmembrane glycoprotein expressed on the cell surface of lymphocytes and other haematopoietic and non-haematopoietic cells [Guan et al., 2009; Hertweck et al., 2011; Di Sante et al., 2013]. CD44 which is also known as phagocytic glycoprotein-1, Hermes antigen, and extracellular matrix receptor type III (ECM III) encompasses a heterogeneous family of receptors with isoforms ranging from 80 to 200 kDa that are encoded by a single gene composed of 19 exons [Gee et al., 2004; Vachon et al., 2006; Iczkowski 2011; Hertweck et al. 2011]. It is a single pass transmembrane glycoprotein involved in cell-cell and cell-matrix adhesion and in cell signalling [Iczkowski 2011]. Diverse functions have been attributed to CD44, including involvement in cellular adhesion and migration, lymphocyte and monocyte homing activation and proliferation, cytotoxic activity of natural killer cells, and tumor metastasis [Vachon et al., 2006].

CD44 is known to be involved in binding, endocytosis, and metabolism of hyaluronan, and has additional functions in innate and adaptive immunity [Vachon et al., 2006]. CD44 is a major adhesion molecule for the extracellular matrix and has been implicated in a wide variety of physiological processes, including lymphocyte homing, activation and proliferation, wound healing, and cell migration, lytic activity of T-cells and NK-cells, as well as in tumor cell invasion and metastasis [Guan et al., 2009; Ishimoto et al., 2011]. It is also implicated in several other cellular processes, such as regulation of growth, survival, differentiation and motility, both under physiologic and pathologic conditions [Di Sante et al., 2013]. In many types of cancers including breast, ovarian and NHL high concentrations of CD44 have been correlated to unfavourable prognosis [Ristamaki et al., 1997; Sillanpää et al., 2003; Louderbough and Schroeder 2011; Chekhun et al., 2013; Di Sante et al., 2013].

6.2 Specific Aims

The aim of this study was to determine,

- The serum concentrations of circulating B-cell activation markers (sCD20, sCD23, sCD27, sCD30, sCD44) in HIV+NHL patients and control populations
- Whether cART has an impact on their serum concentrations

6.3 Materials and Methods

6.3.1 Materials

The following enzyme linked immunosorbent assay (ELISA) kits and reagents were purchased from the Biocom Biotech (Abcam) CC supplier: human sCD23 (Catalogue number: AB119512), sCD30 (Catalogue number: AB113332) and sCD44 (Catalogue number: AB459122). sCD20 (Catalogue number: E14007H) human B-lymphocyte antigen and sCD27 (Catalogue number: EL004910HU) human CD27 antigen ELISA kits were purchased from CUSABIO. Serum separator (SST) tubes (Catalogue number: BD367955) were purchased from the Scientific Group Pty Ltd supplier of Becton Dickinson tubes.

6.3.2 Methods

6.3.2.1 Sample preparation

Blood samples were collected using serum separator tubes (SST) and put on ice immediately. Samples were allowed to clot for 2 hours, and were centrifuged for 15 minutes at 1000xg. Resultant serum was collected and aliquoted into cryo-tubes and was stored at -20°C. Prior to use in the assay, samples were thawed at room temperature and mixed gently. Samples were then diluted according to each individual biomarker protocol provided by the supplier.

6.3.2.2 Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) is a method used for detecting and quantifying a specific protein in a complex mixture [Thermo Scientific 2014]. It enables analysis of protein samples immobilized in micro-plate wells using specific antibodies. The basic principle of ELISA is as follows: i) Coating/Capture: direct or indirect immobilization of antigens to the surface of polystyrene micro-plate wells. ii) Plate Blocking: addition of irrelevant protein or other molecule to cover all unsaturated surface-binding sites of the micro-plate wells. iii) Probing/Detection: incubation with antigen-specific antibodies that affinity-bind to the antigens. iv) Signal measurement: detection of the signal generated via the direct or secondary tag on the specific antibody [Thermo Scientific 2014].

For example, soluble CD23 (sCD23) human in vitro ELISA kit is designed for accurate quantitative measurement of human CD23 concentrations in cell culture supernatant and serum/pasma [Abcam 2013]. This assay employs an antibody specific for human CD23 pre-coated on a 96 well plate. Standards and test samples are added into the wells along with biotinylated CD23 detection antibody and incubated at room temperature. CD23 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and streptavidin-horseradish peroxidase (HRP) conjugate is then added to each well, incubated at room temperature and washed. 3,3',5,5'-Tetramethylbenzidine (TMB) is added and catalyzed by HRP to produce blue colour product that changes into yellow after addition of acidic stop solution. The intensity of the colour is measured at 450nm and

its density is directly proportional to the amount of CD23 sample captured in the plate.

6.3.2.3 Protocol

In the current study, assays for sCD20, sCD23, sCD27, sCD30, and sCD44 were carried out according to the manufacturer's protocols. All followed the basic principles of ELISA. For example, assay for sCD23 was carried out as follows.

6.3.2.3.1 Reagent preparation (sCD23)

All reagents and stored serum samples were equilibrated to room temperature (18-25°C) prior to use. 1x Wash buffer was used to wash the plates, which was prepared by diluting the 20x wash buffer stock concentration 5 fold with distilled water (dH₂O) according to the suppliers' protocol. To make 500ml 1x wash buffer, 50ml 20x wash buffer concentrate was combined with 450 ml dH₂O, mixed thoroughly and gently to avoid foaming and was used immediately, and the remaining wash buffer was stored at 2-8°C for not more than 30 days. 1x Assay Buffer was prepared by diluting the 20x Assay buffer concentrate 5x with dH₂O. 1x Biotin Conjugated antibody was prepared by diluting the anti-Human CD23 monoclonal antibody 100-fold with the prepared 1x-assay buffer and was used within 30 minutes following dilution. 1x Streptavidin-HRP conjugate was prepared by diluting the anti-Streptavidin-HRP conjugate 100 fold with 1x assay buffer.

6.3.2.3.2 Standard preparations (sCD23)

Serial diluted standards were prepared immediately prior to use according to the suppliers protocol. Four hundred U/ml stock standard was prepared by reconstituting one vial of Human CD23 standard with 100µl of dH₂O, and held at room temperature for 10-30 minutes. Seven standards were prepared by adding 225µl sample diluent to all tubes, followed by addition of 225µl of the prepared 400U/ml stock standard to test tube 1. Two hundred U/ml *Standard 2* was prepared by transferring 225µl of the 400Uml Stock standard to test tube 2, mixed thoroughly and gently. Hundred U/ml *Standard 3* was prepared by transferring 225µl from standard 2 to tube 3, mix thoroughly and gently. Fifty U/ml *Standard 4* was also prepared by transferring 225µl from standard 3 to tube 4. Twenty five U/ml *Standard 5* was prepared by transferring 225µl from standard 4 to tube 5 and mixed. 12.3U/ml *Standard 6* was prepared by transferring 225µl from standard 5 to tube 6. *Standard 7* contained sample diluents with no protein added and was the blank control.

6.3.2.3.3 Assay Procedure (sCD23)

All standards, controls and samples were assayed in duplicates. The micro-plate was washed twice with approximately 400µl 1x wash buffer per well with thorough aspiration of micro-plate contents between washes. The 1x wash buffer was allowed to remain in the wells for about 10-15 seconds before aspiration. Following the last wash step, the wells were emptied and the micro-plate was tapped on paper towel to remove excess 1x wash buffer. Micro-plate strips were used immediately after washing. Hundred µl of prepared standards (including the standard blank control) and samples were added to the appropriate wells in duplicates. Fifty µl of sample diluent was added to all sample wells (not to standards), followed by the addition of 50µl of sample diluent Biotin-Conjugate. The micro-plate was covered with adhesive film and incubated at room temperature (18-25°C) for 2 hours on a shaker set at 100rpm.

The adhesive film was removed, wells emptied and washed 3 times with approximately 400µl 1x Wash buffer per well with thorough aspiration of micro-plate contents between washes. Hundred µl of Streptavidin-HRP was added to all wells, plate covered with adhesive film and incubated at room temperature for 2 hours on shaker. The adhesive film was removed, wells emptied and washed 3 times as described previously. Hundred µl of TMB substrate solution was added to all wells and the micro-plate was incubated at room temperature for 10 minutes on a shaker set at 400rpm. Colour development was monitored and the substrate reaction was stopped by adding 100µl of stop solution into all wells before signal in the positive wells becomes saturated.

The absorbance of each micro-plate was read on a spectrophotometer using 450nm as the primary wave length. The plate reader was blanked using the blank wells and the absorbance was determined for both the samples and standards.

6.3.2.3.4 Calculations

The duplicate readings were averaged for each standard, sample and blank control. The blank control was subtracted from all mean readings and the mean standard readings were plotted against their concentrations to construct a standard curve. The protein concentrations for unknown and control samples were extrapolated from the standard curve plotted.

6.4 Results

6.4.1 Serum concentrations of circulating soluble CD20 (sCD20)

The serum concentrations of circulating sCD20 were significantly higher in HIV positive NHL (HIV+NHL) patients as compared to HIV negative NHL (NHL) patients (5.62 ± 1.69 vs 3.92 ± 0.63 ; $p < 0.0001$) as well as when compared to HIV positive patients on a cART regimen (HIV+ cART) (5.62 ± 1.69 vs 4.75 ± 1.34 ; $p = 0.0359$) (figure 6.1). NHL patients had significantly high serum concentrations of circulating sCD20 than controls (3.92 ± 0.63 vs 3.04 ± 0.84 ; $p = 0.0025$). There was no significant difference in the serum concentrations of circulating sCD20 between HIV+ cART and cART-naïve HIV+ patients. However, cART-naïve HIV+ patients had higher serum concentrations of circulating sCD20 than controls (5.11 ± 1.49 vs 3.04 ± 0.84 ; $p < 0.0001$).

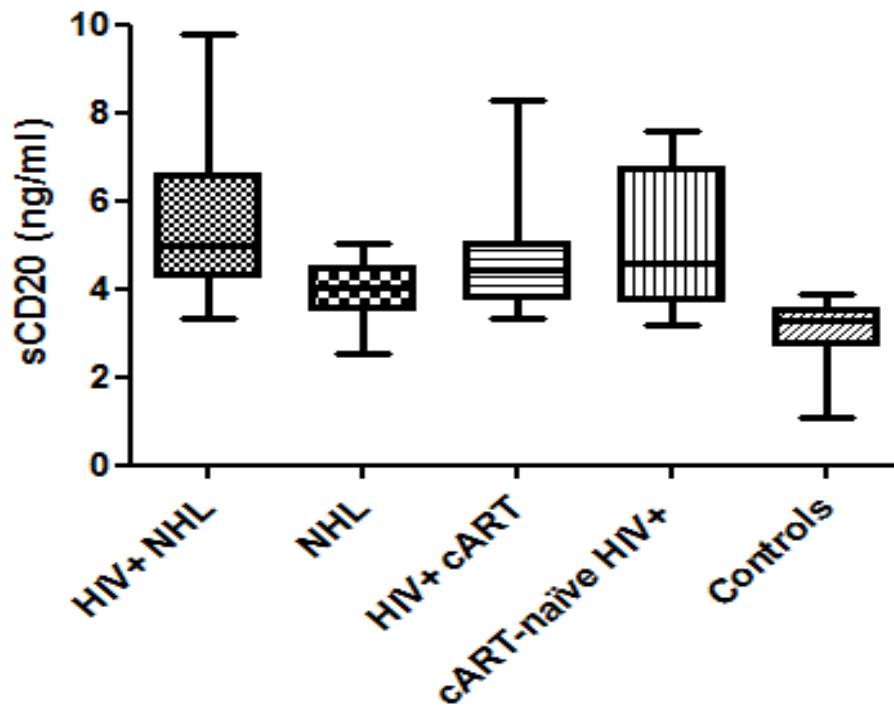


Figure 6.1 Serum concentrations of circulating soluble CD20 (sCD20). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

6.4.1.1 Soluble CD20 (sCD20) correlations

The serum concentrations of circulating sCD20 were negatively correlated with the numbers of CD4 T-cells ($r=-0.3208$, $p<0.0001$), NKT-cells ($r=-0.1701$, $p=0.0219$), and FoxP3 expression ($r=-0.3604$, $p<0.0001$) (figure 6.2A, C, and E), while these serum concentrations of circulating sCD20 were positively correlated with the numbers of NK-cells ($r=0.2261$, $p=0.0035$), and CD8+CD38 expression ($r=0.172$, $p=0.0203$) (figure 6.2B and D). Following adjustment for age, gender, smoking status, viral load and duration of treatment, the serum concentrations of sCD20 were independently associated with HIV+NHL (OR = 1.79; 95% CI = 0.69–4.63).

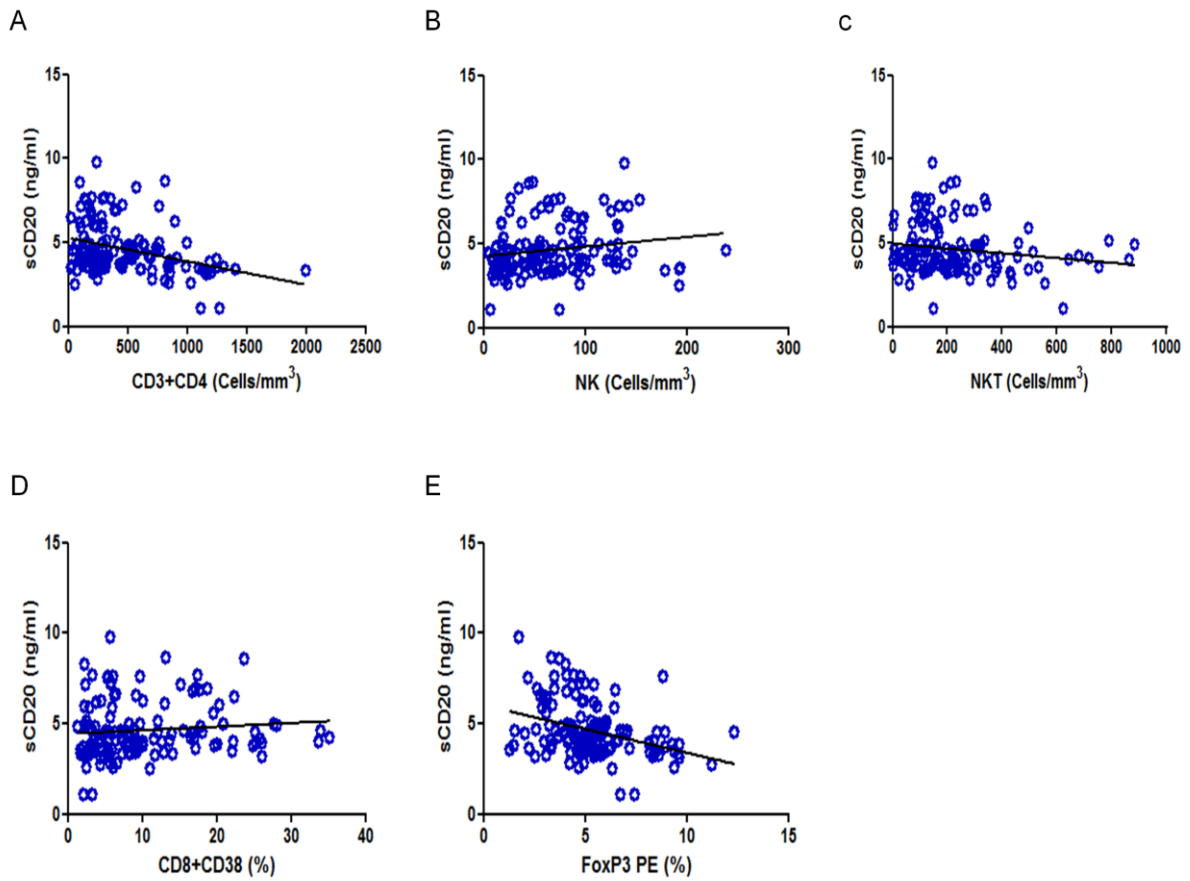


Figure 6.2 Soluble CD20 (sCD20) Correlations. A) CD4 vs sCD20; B) NK vs sCD20; C) NKT cells vs sCD20 D) CD8+CD38 vs sCD20 E) FoxP3 vs sCD20. NK, Natural killer cells; NKT, Natural killer T-cells, FoxP3, Forkhead box protein 3.

6.4.2 Serum concentrations of circulating soluble CD23 (sCD23)

The serum concentrations of circulating sCD23 were significantly higher in HIV positive NHL (HIV+NHL) patients as compared to both HIV negative NHL (NHL) patients (204.63 ± 92.12 vs 148.76 ± 94.28 ; $p=0.0192$) and HIV positive patients on a cART regimen (HIV+ cART) (204.63 ± 92.12 vs 139.20 ± 130.83 ; $p<0.0001$) (figure 6.3). However, NHL patients had significantly high serum concentrations of circulating sCD23 than controls (148.76 ± 94.28 vs 94.32 ± 35.54 ; $p=0.0178$). The serum concentrations of circulating sCD23 were significantly higher in HIV+ cART patients as compared to cART-naïve HIV+ patients (139.20 ± 130.83 vs 69.28 ± 48.90 ; $p=0.0074$). As compared to the controls, the serum concentrations of circulating sCD23 were significantly lower in cART-naïve HIV+ patients (69.28 ± 48.90 vs 94.32 ± 35.54 ; $p=0.0452$).

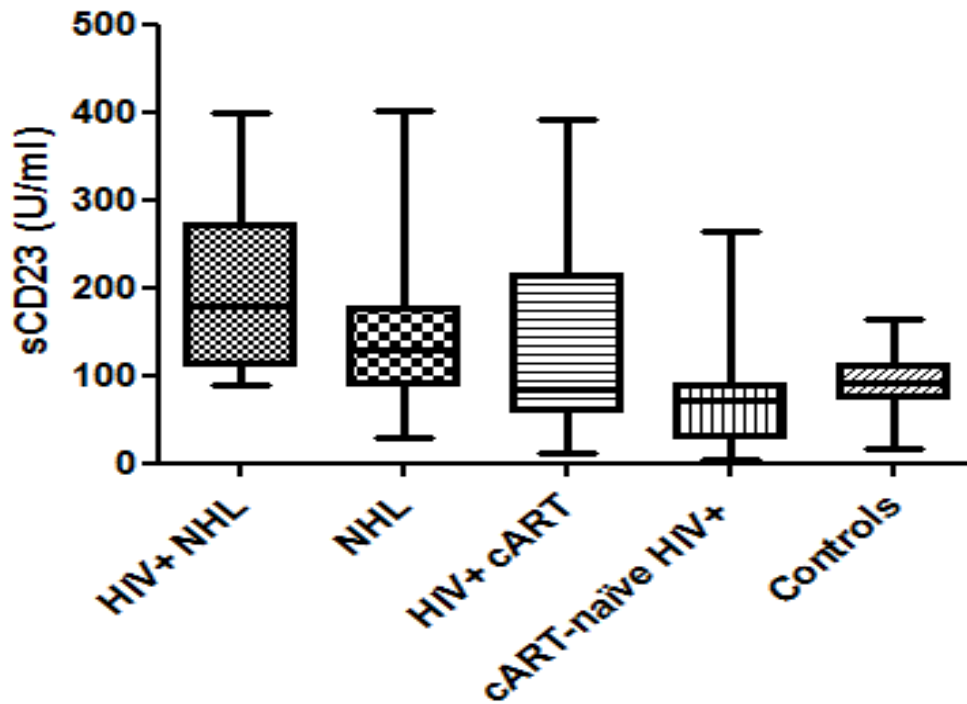


Figure 6.3 Serum concentrations of circulating soluble CD23 (sCD23). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

6.4.2.1 Soluble CD23 (sCD23) correlations

The serum concentrations of circulating sCD23 were positively correlated with the numbers of NK-cells ($r=0.1976$, $p=0.0094$) (figure 6.4). There was no correlation between the serum concentrations of circulating sCD23 and the expression of other cell types. Following adjustment for age, gender, smoking status, viral load and duration of treatment, the serum concentrations of sCD23 were independently associated with HIV+NHL (OR = 1.13; 95% CI = 0.99–1.3).

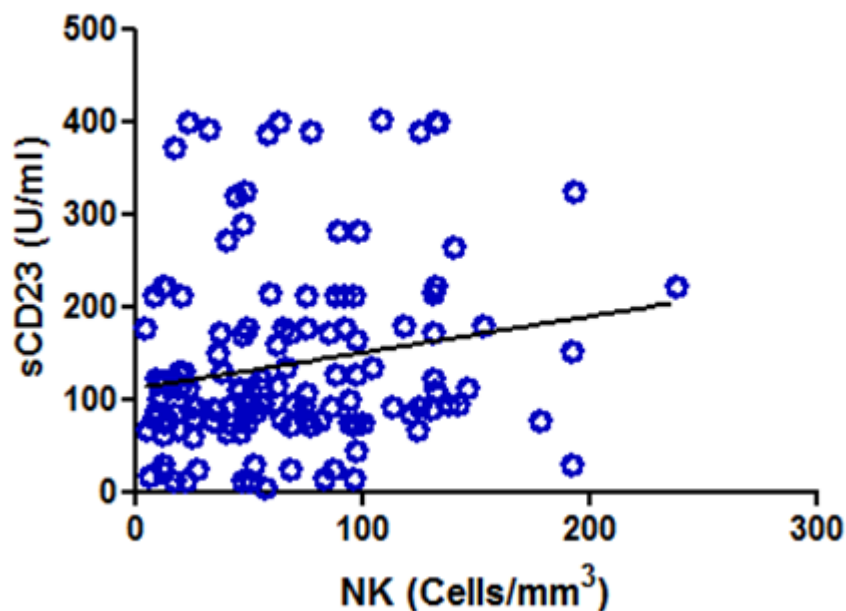


Figure 6.4 Soluble CD23 (sCD23) Correlations. A) NK cells vs sCD23. NK, Natural killer.

6.4.3 Serum concentrations of circulating soluble CD27 (sCD27)

There was no significant difference in the serum concentrations of circulating sCD27 when HIV positive NHL (HIV+NHL) patients were compared to HIV negative NHL (NHL) patients. However, as compared to HIV positive patients on a cART regimen (HIV+ cART), the serum concentrations of circulating sCD27 were significantly higher in HIV+NHL patients (22.80 ± 11.20 vs 13.71 ± 4.09 ; $p=0.0007$) (figure 6.5). The serum concentrations of circulating sCD27 were significantly higher in NHL when compared to the controls (22.28 ± 12.87 vs 12.21 ± 1.87 ; $p=0.0033$). HIV+ cART patients had significantly lower serum concentrations of circulating sCD27 as compared to cART-naïve HIV+ (13.71 ± 4.09 vs 19.74 ± 9.48 ; $p=0.0038$) patients. cART-naïve HIV+ patients had higher serum concentrations of circulating sCD27 than the controls (19.74 ± 9.48 vs 12.21 ± 1.87 ; $p=0.0025$).

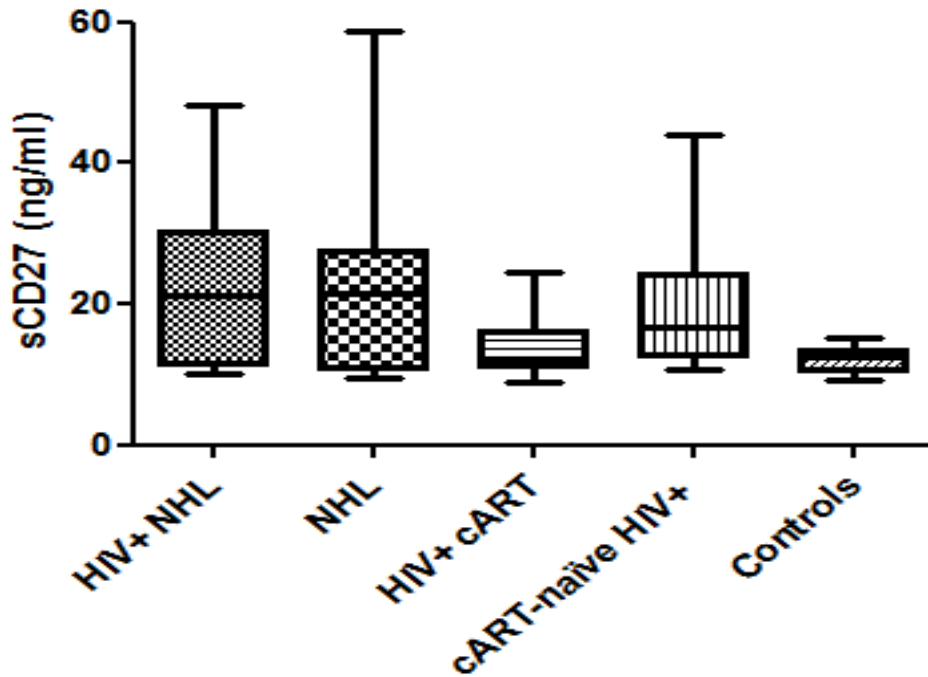


Figure 6.5 Serum concentrations of circulating soluble CD27 (sCD27). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

6.4.3.1 Soluble CD27 (sCD27) correlations

The serum concentrations of circulating sCD27 were negatively correlated with the numbers of CD4 T-cells ($r=-0.3200$, $p<0.0001$), and FoxP3 expression ($r=-0.164$, $p=0.0260$) (figure 6.6A and C). However, these serum concentrations of circulating sCD27 were positively associated with CD8+CD38 ($r=0.201$, $p=0.0082$) (figure 6.6B) expression. Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association between sCD27 and HIV+NHL was observed.

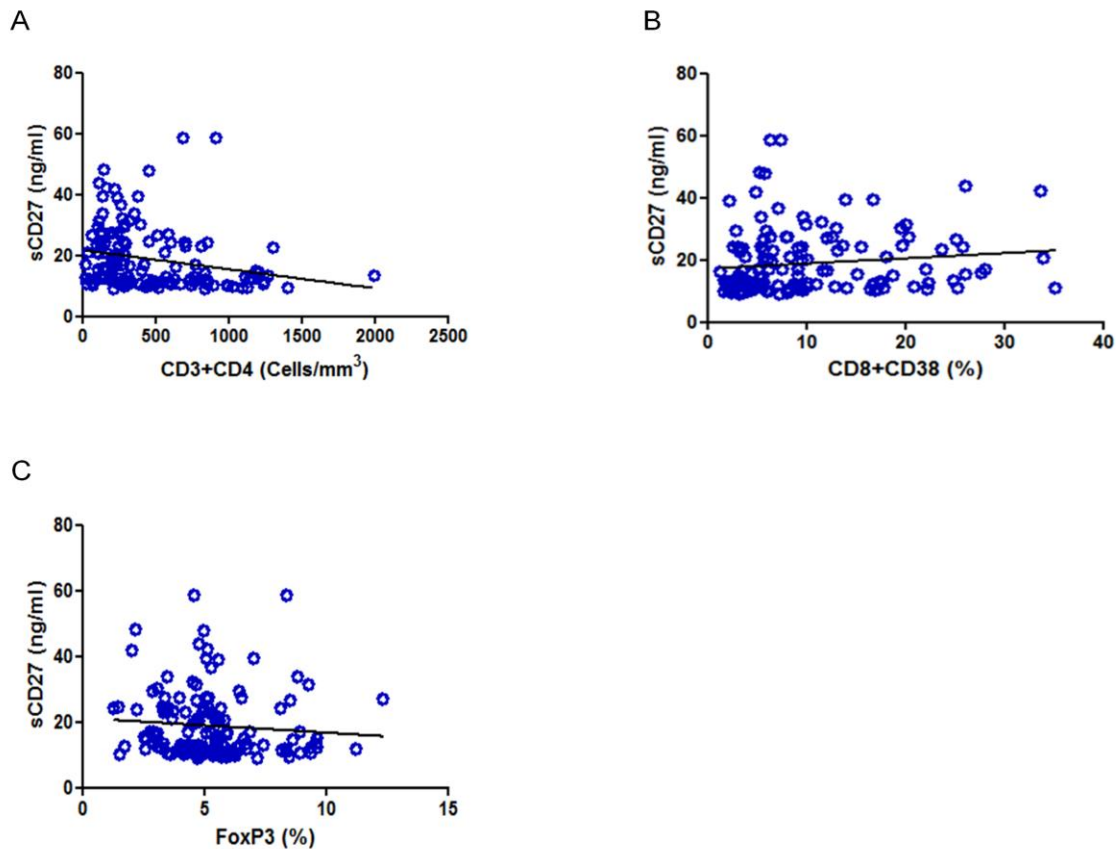


Figure 6.6 Soluble CD27 (sCD27) Correlations. A) CD4 vs sCD27 B) CD8+CD38 vs CD27 C) FoxP3 vs CD27. FoxP3, Forkhead box protein 3.

6.4.4 Serum concentrations of circulating soluble CD30 (sCD30)

The serum concentrations of circulating sCD30 were significantly higher in HIV positive NHL (HIV+NHL) patients as compared to both HIV negative NHL (NHL) (577.41 ± 256.31 vs 384.50 ± 176.07 ; $p=0.0052$) and HIV positive patients on a cART regimen (HIV+ cART) (577.41 ± 256.31 vs 274.11 ± 266.13 ; $p<0.0001$) (figure 6.7). As compared to the controls, NHL patients had significantly higher serum concentrations of circulating sCD30 (384.50 ± 176.07 vs 239.98 ± 117.04 ; $p=0.0078$). There was no significant difference in the serum concentrations of circulating sCD30 between HIV+ cART and cART-naïve HIV+ patients as well as between cART-naïve HIV+ patients and controls.

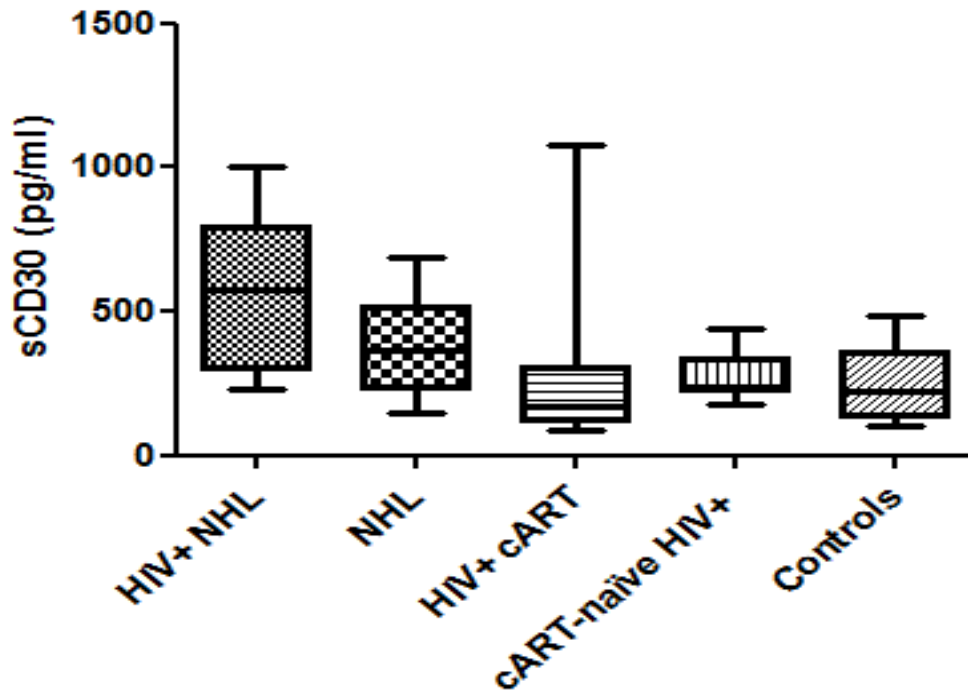


Figure 6.7 Serum concentrations of circulating soluble CD30 (sCD30). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

6.4.4.1 Soluble CD30 (sCD30) correlations

The serum concentrations of circulating sCD30 were negatively correlated with the numbers of CD4 T-cells ($r=-0.1582$, $p=0.0305$) (figure 6.8). There was no significant correlation between sCD30 and other cell types. Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association between sCD30 and HIV+NHL was observed.

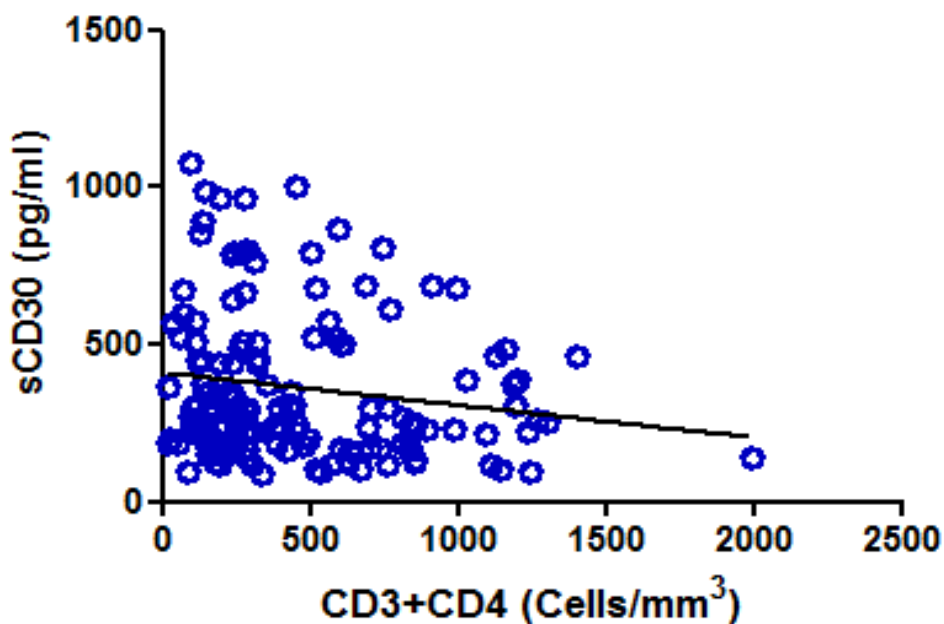


Figure 6.8 Soluble CD30 (sCD30) Correlations. CD3+CD4 vs sCD30 expression

6.4.5 Serum concentrations of circulating soluble CD44 (sCD44)

The serum concentrations of circulating sCD44 were significantly higher in HIV positive NHL (HIV+NHL) patients as compared to both HIV negative NHL (NHL) patients (7.25 ± 1.23 vs 6.03 ± 1.41 ; $p=0.0014$) and HIV positive patients on a cART regimen (HIV+ cART) (7.25 ± 1.23 vs 4.84 ± 1.57 ; $p<0.0001$) (figure 6.9). As compared to the control population, NHL patients had significantly higher serum concentrations of circulating sCD44 (6.03 ± 1.41 vs 4.30 ± 1.37 ; $p=0.0013$). HIV+ cART patients had significantly lower serum concentrations of circulating sCD44 than cART-naïve HIV+ patients (4.84 ± 1.57 vs 6.08 ± 2.61 ; $p=0.0130$). cART-naïve HIV+ patients had higher serum concentrations of circulating sCD44 than controls (6.08 ± 2.61 vs 4.30 ± 1.37 ; $p=0.0030$).

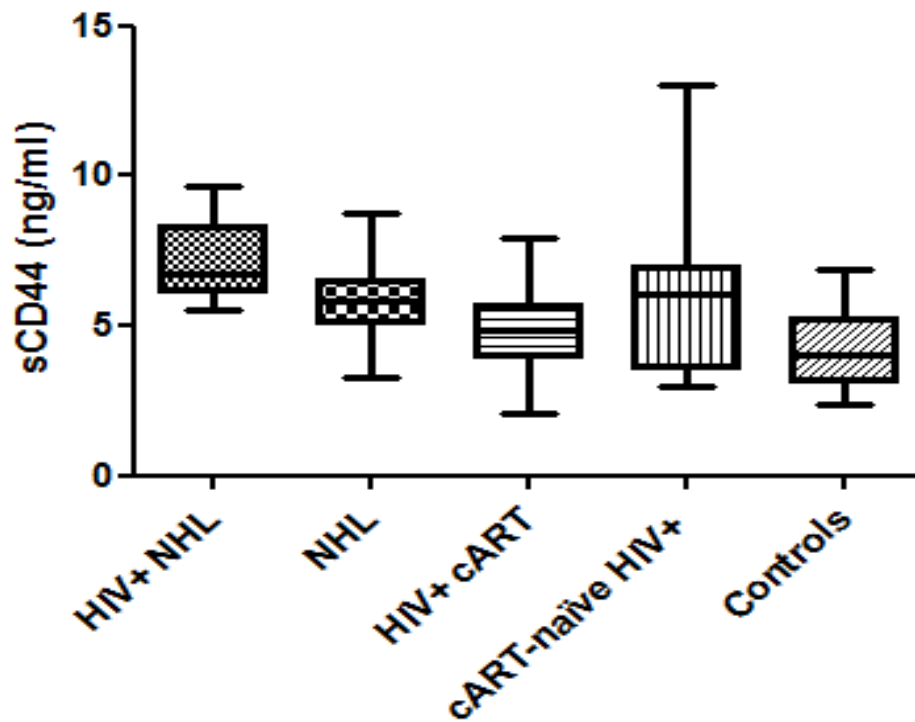


Figure 6.9 Serum concentrations of circulating soluble CD44 (sCD44). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

6.4.5.1 Soluble CD44 (sCD44) correlations

The serum concentrations of circulating sCD44 were negatively correlated with the numbers of CD4 T-cells ($r=-0.2848$, $p=0.0003$) and NKT-cells ($r=-0.1681$, $p=0.0232$) (figure 6.10A and C). However, these serum concentrations of circulating sCD44 were positively associated with the numbers of NK-cells ($r=0.1554$, $p=0.0329$) and CD8+CD38 ($r=0.1676$, $p=0.0235$) expression (figure 6.10B and D). Following adjustment for age, gender, smoking status, viral load and duration of treatment, the serum concentrations of sCD44 were independently associated with HIV+NHL (OR = 5.97; 95% CI = 1.52–23.33).

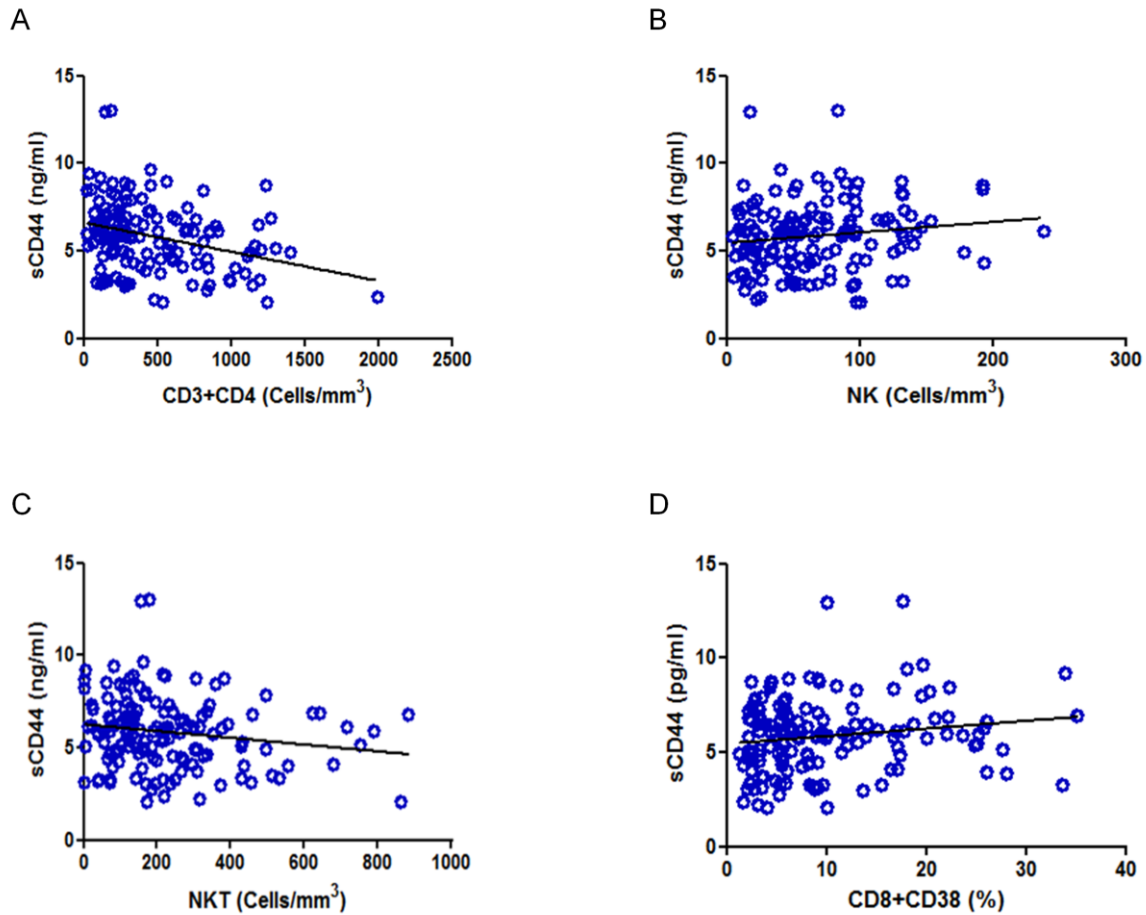


Figure 6.10 Soluble CD44 (sCD44) Correlations. A) CD4 vs sCD44 B) NK cells vs sCD44 C) NKT cells vs sCD44 D) CD8+CD38 vs sCD44. NK, Natural killer cells; NKT, Natural killer T-cells.

6.5 Discussion

As HIV-1 infection leads to chronic immune activation, the serum concentrations of circulating B-cell activation markers were investigated in the current study. B-cell activation has been previously suggested to play a role in the development of HIV+NHL as shown by the biomarkers of immune activation. However, this has not been investigated in our patient population. In the current study, the serum concentrations of circulating sCD20, sCD23, sCD27, sCD30 and sCD44 were studied.

6.5.1 Soluble CD20 (sCD20)

CD20 is expressed in more than 90% of B cell lymphoma cells and has become a molecular target for monoclonal antibody therapeutics [Tokunaga et al., 2014]. Previous studies have shown increased expression of sCD20 in HIV+NHL and chimeric antibody against sCD20 (rituximab) has been included as part of chemotherapy [Sparano et al., 2010]. In the current study, the serum concentrations of circulating sCD20 were significantly elevated in HIV+NHL as compared to NHL, as well as when compared to HIV+ cART patients (figure 6.1). These results confirm that the serum concentrations of circulating sCD20 are indeed up-regulated in HIV+NHL patients and may reflect the B-cell activation observed in this patient group. The serum concentrations of circulating sCD20 were increased in NHL patients as compared to the controls (figure 6.1). In addition, the effect of cART on the serum concentrations of circulating sCD20 was studied. No statistical significant difference in serum concentrations of circulating sCD20 between HIV+ cART and cART-naïve HIV+ patients was found. This suggests that cART may have no effect

on the serum concentrations of circulating sCD20. However, the serum concentrations of circulating sCD20 are increased in untreated HIV-1 infection.

To investigate whether HIV influenced the serum concentrations of circulating sCD20, cART-naïve HIV+ patients were compared with the control populations. The serum concentrations of circulating sCD20 were significantly higher in cART-naïve HIV+ patients as compared to the controls. This suggests that HIV-1 infection increases the serum concentrations of circulating sCD20. It has been shown that sCD20 expression is higher in more advanced HIV diseases [Staal et al., 1992].

The elevated serum concentrations of circulating sCD20 were negatively associated with the numbers of CD4 T-cells, NKT-cells and FoxP3 expression (figure 6.2A, C, and E), all of which are down-regulated by HIV-1 infection. Staal and colleagues [1992], reported that sCD20 expression tends to be highest in HIV-1 infected patients who have the fewest CD4 T-cell count. Furthermore, the serum concentrations of circulating sCD20 were positively correlated with the numbers of NK-cells and CD8+CD38 expression (figure 6.2B and D). In addition, the serum concentrations of sCD20 were independently associated with HIV+NHL. This data suggests that serum concentrations of circulating sCD20 are elevated in HIV+NHL and this may contribute to chronic B cell activation observed in HIV+NHL and untreated HIV+ patients.

6.5.2 Soluble CD23 (sCD23)

As mentioned previously, sCD23 plays a role in survival extension of B-cells and the induction of cell growth and differentiation of B, myeloid and T-cells [Lopez-Matas et al., 2000]. Therefore sCD23 may play a role in the development of B-cell malignancies such as DLBCL. Serum sCD23 concentrations have been shown to be elevated prior to the diagnosis of HIV+NHL and they may serve as a predictive marker for the development of HIV+NHL in HIV-1 infected patients [Hennig et al., 1998; Schroeder et al., 1999]. In the current study, the serum concentrations of circulating sCD23 were significantly higher in HIV+NHL as compared to NHL as well as when compared to HIV+ cART patients (figure 6.3). These results suggest that sCD23 is increased in HIV+NHL and are consistent with previous studies.

The elevated serum concentrations of circulating sCD23 observed in the current study may have been caused by persistent infection by HIV-1 virus which causes immune activation. This has been confirmed by increased serum concentrations of circulating sCD23 in NHL when compared to a control population. However, in a multicentre HIV cohort study, Schroeder and colleagues [1999], showed elevated serum sCD23 concentrations in HIV+NHL, and these concentrations were not mediated by EBV in these patients. In a group of 134 patients, Bossolasco and colleagues [2001] reported higher concentrations of sCD23 in the cerebrospinal fluid from HIV+NHL patients with brain involvement. In contrast, Jarrin and colleagues [2011], reported that elevated serum concentrations of sCD23 did not predict the clinical appearance of HIV+NHL, but the expression of sCD23 at diagnosis was elevated as compared with controls.

In addition, the serum concentrations of circulating sCD23 were significantly increased in HIV+ cART than cART-naïve HIV+ patients in the current study (figure 6.3). Thus, cART did not have an impact on the serum concentrations of circulating sCD23 in the current study. In contrast, Yawetz and colleagues [1995], showed that serum sCD23 concentrations were elevated in HIV-1 infected patients who developed HIV+NHL as compared to treated HIV+ patients and healthy controls. Thus, the observed increased serum concentrations of circulating sCD23 in cART-naïve HIV+ patients may contribute to lymphomagenesis. Furthermore, the elevated serum concentrations of circulating sCD23 were positively associated with NK-cells which may play a role in eradication of lymphoma cells. In addition, the serum concentrations of sCD23 were independently associated with HIV+NHL. These findings confirm the association between HIV-1 infection with increased serum concentrations of circulating sCD23.

6.5.3 Soluble CD27 (sCD27)

sCD27 plays an important role in the activation, proliferation, and survival of T-cells, B-cell clonal expansion as well as in NK-cell cytolytic activity and has been used routinely as a marker of memory B-cells [Siedel 2012]. sCD27 is a biomarker associated with both T and B-cell activation [Najafi et al., 2013], thus increased serum concentrations of sCD27 in HIV+NHL may be expected as increased B-cell activation were previously reported in this patient population. In the current study, there was no significant difference in the serum concentrations of circulating sCD27 between HIV+NHL and NHL. However, the serum concentrations of circulating sCD27 were significantly higher in HIV+NHL when compared to HIV+ cART patients

(figure 6.5). In addition, serum concentrations of circulating sCD27 were significantly increased in NHL as compared to controls. These findings suggest that the serum concentrations of circulating sCD27 are elevated in NHL regardless of the HIV status. However, the HIV-1 infection did not influence the serum concentrations of circulating sCD27 within the lymphoma groups. Widney and colleagues [1999], reported that serum sCD27 concentrations in HIV+NHL were twice as high as the concentrations seen in treated HIV-1 infected patients and controls. Furthermore, Goto and colleagues [2012], demonstrated that elevated serum sCD27 concentrations are associated with poor outcome in DLBCL patients. In the current study, the serum concentrations of circulating sCD27 were significantly lower in HIV+ cART as compared to cART-naïve HIV+ patients (figure 6.5).

The serum concentrations of circulating sCD27 were elevated in cART-naïve HIV+ patients as compared to the controls. The increased serum concentrations of circulating sCD27 may have been caused by chronic immune activation which is a hallmark feature of HIV-1 infection. These findings suggest that the serum concentrations of circulating sCD27 are elevated in HIV+ patients and cART may partially decrease them. In addition, the serum concentrations of circulating sCD7 were negatively associated with CD4 T-cell counts and FoxP3 expression (figure 6.6A and C), while they correlated positively with CD8+CD38 expression (figure 6.6B). These findings confirm the association between HIV-1 infection and T-cell activation with increased serum sCD27 concentrations. The increased immune activation may have led to decreased CD4 T-cell counts and FoxP3 expression. T-

cell activation which is the hallmark feature of HIV-1 infection, may have contributed to the observed elevated serum concentrations of circulating sCD27.

6.5.4 Soluble CD30 (sCD30)

sCD30 is expressed in activated B, T and NK-cells, thus its expression may be increased in HIV+NHL. In the current study, the serum concentrations of circulating sCD30 were significantly up-regulated in HIV+NHL as compared to both NHL and HIV+ cART patients (figure 6.7). In addition, the serum concentrations of circulating sCD30 were significantly increased in NHL patients as compared to the controls (figure 6.7). These results suggest that sCD30 is increased in HIV+NHL and HIV negative NHL. Thus, the serum concentrations of circulating sCD30 are increased in NHL patients regardless of the HIV status. It has been reported that the serum concentrations of sCD30 increases in some neoplastic diseases as the result of release by neoplastic and reactive cells expressing CD30 [Horie and Watanabe 1998]. In a cross sectional study, Breen and colleagues [2006], reported elevated serum sCD30 concentrations in HIV+NHL as compared to HIV+ cART and healthy controls.

In a prospective study, Purdue and colleagues [2009], showed that serum sCD30 concentrations were 39% higher in NHL patients as compared to healthy controls. In the current study, there was no significant difference in the serum concentrations of circulating sCD30 between HIV+ cART and cART-naïve HIV+ patients (figure 6.7). HIV-1 infection was not associated with the serum concentrations of circulating sCD30 in the current study and this is in agreement with the lack of significant

difference between cART-naïve HIV+ patients and controls (figure 6.7). However, the serum concentrations of circulating sCD30 were negatively associated with CD4 T-cell counts. It has been shown that sCD30 expression and stimulation may play an important role in both HIV-1 replication and the death of HIV-1 infected CD4 T-cells [Romagnani et al., 1996]. As mentioned previously, sCD30 is expressed in activated T and B-cells, thus the increased serum concentrations of circulating sCD30 that were observed in the current study reflect the increased T and B-cell activation which is also confirmed by the negative correlation with CD4 T-cell count. The increased serum concentrations of circulating sCD30 have been observed in HIV-1 infected patients and were associated with a more rapid disease progression and poor survival [Horie and Watanabe 1998].

6.5.5 Soluble CD44 (sCD44)

sCD44 has been previously implicated in tumor metastasis and progression [Jothy 2003; Marhaba and Zoller 2004; Heyse et al., 2010]. In addition, sCD44 is involved in the development and progress of haematological neoplasia by enhancement of apoptotic resistance, invasiveness, as well as regulation of bone marrow homing, and mobilization of leukemia initiating cells in the peripheral blood [Hertweck et al., 2011]. The altered sCD44 expression has been used as a marker of poor prognosis in most haematological malignancies [Hertweck et al., 2011]. The serum concentrations of circulating sCD44 were greatly increased in HIV+NHL as compared to both NHL and HIV+ cART patients in the current study (figure 6.9). These findings suggest that serum concentrations of circulating sCD44 are elevated

in HIV+NHL. Breen and colleagues [2005], showed that serum concentrations of sCD44 are significantly elevated in HIV+ patients who went on to develop HIV+NHL. It has been reported that elevated serum sCD44 concentrations at diagnosis is associated with a high IPI score, poor response to treatment, and unfavourable outcome in NHL [Ristamaki et al., 1997; Navarro et al., 2000]. In addition, serum concentrations of circulating sCD44 were elevated in NHL as compared to controls in the current study (figure 6.9). This confirms the increased serum sCD44 concentrations in NHL regardless of the HIV status and this may result in an unfavourable outcome. However, HIV+ cART patients had significantly lower serum concentrations of circulating sCD44 than cART-naïve HIV+ patients (figure 6.9). In addition, the serum concentrations of circulating sCD44 were significantly increased in cART-naïve HIV+ patients as compared to the controls (figure 6.9).

These results suggest that the serum concentrations of circulating sCD44 are greatly increased in HIV+ patients, and cART may reduce the expression of sCD44 in HIV+ patients. The observed high serum concentrations of circulating sCD44 in HIV+NHL may have been caused by HIV-1 infection. In addition, the serum concentrations of circulating sCD44 were negatively associated with the numbers of CD4 T-cells and NKT-cells (figure 6.10A and C) in the current study. However, the serum concentrations of circulating sCD44 were positively associated with the numbers of NK-cells and CD8 T-cell activation. In addition, the serum concentrations of sCD44 were independently associated with HIV+NHL. The increased T-cell activation occurring in HIV+ patients may have led to increased serum concentrations of circulating sCD44.

6.6 Conclusion

The serum concentrations of circulating B-cell activation markers are elevated in HIV+NHL patients and cART may decrease them. B-cell activation is increased in HIV+NHL as evidenced by increased B-cell activation markers investigated in this study, and is associated with decreased CD4 T-cell counts and increased T-cell activation. The serum concentrations of sCD20, sCD23 and sCD44 are independently associated with HIV+NHL. These findings confirm that B-cell activation is increased in untreated HIV+ patients and in NHL patients. The increased immune activation in this patient population group may have been caused by persistent HIV-1 infection, as well as suppressed immune regulation.

CHAPTER SEVEN

PRO-INFLAMMATORY CYTOKINES

7.1 Introduction

Inflammation is a co-ordinated process induced by microbial infection or tissue injury [Barton 2008]. It is an adaptive response that is triggered by noxious stimuli and conditions whose main function is to resolve the infection or repair the damage and return to a state of homeostasis [Barton 2008; Medzhitov 2008]. Inflammation can occur in response to dietary or environmental factors, infection, and autoimmune diseases [Beyaert et al., 2013]. The inflammatory response to infection has traditionally been classified as 4 distinct phases i.e. recognition of infection, recruitment of cells to the site of infection, elimination of the microbe, and resolution of inflammation and return to homeostasis [Barton 2008].

The inflammatory response triggered by infection precedes tumor development and is part of the normal host defence against pathogens [Grivennikov et al., 2010]. Tumor micro-environment contains innate immune cells (including macrophages, neutrophils, mast cells, myeloid derived suppressor cells, dendritic cells, and NK-cells) and adaptive immune cells (T and B lymphocytes) in addition to the cancer cells and their surrounding stroma [Balkwill and Mantovani 2001; Grivennikov et al., 2010]. Inflammation is recognized as a hallmark feature of cancer development and progression [Diakos et al., 2014]. The hallmarks of cancer related inflammation include the presence of inflammatory cells and inflammatory mediators (such as chemokines, cytokines and prostaglandins) in tumor tissues, tissue remodelling and

angiogenesis similar to that seen in chronic inflammatory responses and tissue repair [Mantovani et al., 2008]. The connections between inflammation and cancer consist of two pathways i.e. i) Extrinsic pathway which is driven by inflammatory conditions that increase cancer risk (such as inflammatory bowel disease); ii) Intrinsic pathway which is driven by genetic alterations that cause inflammation and neoplasia (such as oncogenes) [Balkwill and Mantovani 2001; Mantovani et al., 2008]. Chronic inflammation can initiate tumorigenesis by directly causing DNA alterations or making cells more susceptible to mutagens [Beyaert et al., 2013].

Persistent infections within the host, induce chronic inflammation which can result in leukocytes and other phagocytic cells to induce DNA damage in proliferating cells, through their generation of reactive oxygen and nitrogen species that are normally produced by these cells to fight infections [Coussens and Werb 2002; Grivennikov and Karin 2010]. These species react to form peroxynitrite, a mutagenic agent that causes genomic alterations such as point mutations, deletions, or rearrangements [Coussens and Werb 2002]. Oncogene activation or cell senescence induced by DNA damage or oncogene activation can enhance the transcription of pro-inflammatory genes, coding for cytokines and chemokines [Grivennikov and Karin 2010]. There is evidence that local inflammatory processes and antigenic drive can promote lymphoma development at the site of inflammation/immune activation [Baecklund et al., 2014].

Inflammatory cytokines produced by tumor infiltrating immune cells, such as IL-6 and tumor necrosis factor- α (TNF- α) can serve as mitogens and survival factors for pre-malignant and fully established cancer cells [Grivennikov and Karin 2010]. Inflammation also contributes to the induction of angiogenesis, which is critical for supplying the growing tumor with necessary nutrients and oxygen [Grivennikov and Karin 2010]. Much of the growth stimulating cross-talk between immune and malignant cells is mediated by cytokines that activate the oncogenic transcription factors NF- κ B and STAT3 [Grivennikov and Karin 2010]. Cytokines released by the chronic inflammatory cells, such as IL-10 which inhibits T-cell proliferation, may contribute to the proliferation of EBV infected B-cells which subsequently accumulate genetic alterations, undergo clonal selections and eventually evolve into lymphoma [Baecklund et al., 2014].

7.1.1 Cytokines

Cytokines are a group of low molecular weight, soluble proteins that arise from one cell and which affect and regulate the activity of immune cells by transmitting intercellular signals [Leonard and Lin 2000]. Cytokines are produced by many cell populations, but they are predominantly produced by helper T-cells and macrophages [Zhang and An 2007]. They may act on the cells that secrete them (autocrine), on nearby cells (paracrine), or in some instances on distant cells (endocrine) [Zhang and An 2007]. Cytokines control a variety of important biological responses related to haematopoiesis and immune function [Imada and Leonard 2000]. They control growth, differentiation, and other functions of immune and haematopoietic cells [Yasukawa et al., 2000; Imada and Leonard 2000].

Cytokines play an essential role in the development, differentiation, and function of myeloid and lymphoid cells [Tamiya et al., 2011]. Over the years, the signalling pathways induced by these cytokines have been extensively studied, including Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway (figure 7.1) [Imada and Leonard 2000; Shuai and Liu 2003]. JAK/STAT pathway plays an essential role in driving biological responses to cytokines [Imada and Leonard 2000]. In this pathway, cytokine binding results in receptor oligomerization, which initiates the activation of JAK tyrosine kinases (JAK1, JAK2, JAK3, and Tyk2) which in turn phosphorylate STATs, leading to a rapid signalling and translocation from the cell surface to the nucleus where they activate gene transcription [Imada and Leonard 2000; Shuai and Liu 2003; Tamiya et al., 2011].

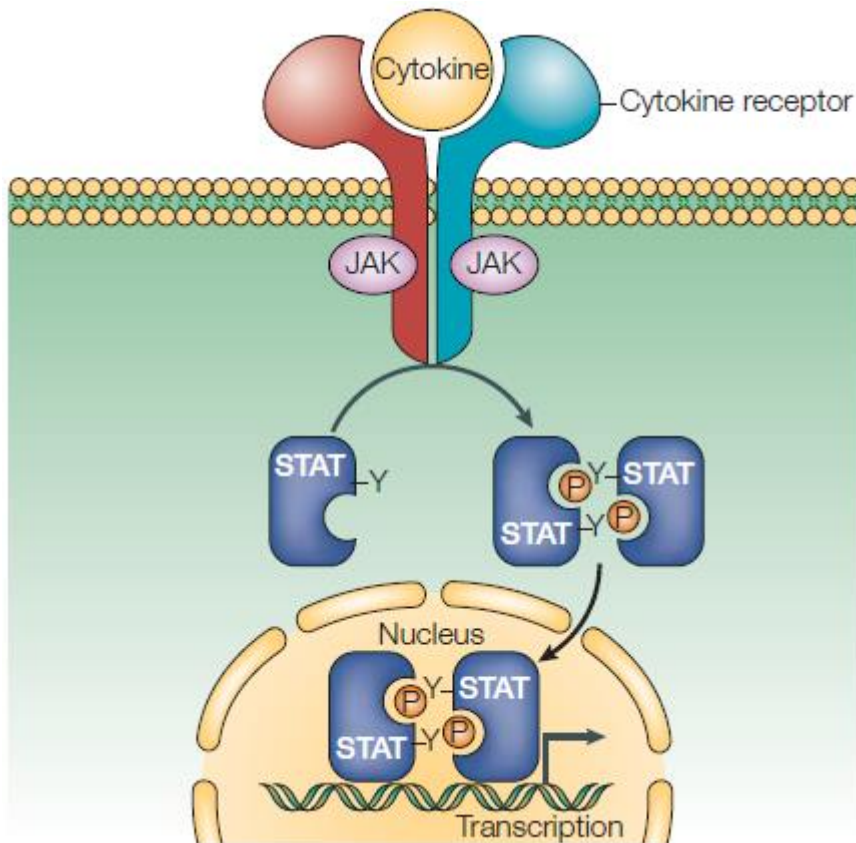


Figure 7.1: Schematic representation of the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway. The activation of JAKs after cytokine stimulation results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription. [From: Shuai and Liu 2003].

Cytokines can be classified into two major groups based on their functional characteristics i.e. T_H1 and T_H2 cytokines [Leonard and Lin 2000; Coussens and Werb 2002]. T_H1 cytokines include among others IL-1, IL-2, IFN- γ , TNF- α , and T_H2 cytokines include IL-4, IL-6, IL-10 and IL-13 (figure 7.2) [Leonard and Lin 2000; Coussens and Werb 2002]. The primary function of cytokines is the mediation and regulation of immunity, inflammation and haematopoiesis [Myers 2009]. Some

cytokines promote inflammation and are called pro-inflammatory cytokines, whereas others suppress the activity of pro-inflammatory cytokines and are known as anti-inflammatory cytokines (figure 7.2) [Dinarello 2000; Zhang and An 2007]. The imbalance in the concentrations of these cytokines may result in tumour growth or regression [Coussens and Werb 2002]. Inflammatory cells are present in most solid tumors [Beyaert et al., 2013]. These cells promote tumor cell survival, proliferation, and dissemination, and high concentrations are associated with a poor prognosis [Beyaert et al., 2013].

Pro-inflammatory cytokines are associated with tumor invasion and progressive disease and are released in response to many antineoplastic agents [Myers 2009]. Inflammatory mediators, including cytokines such as TNF- α , IL-1 and IL-6, growth factors, chemokines, and proteases produced by tumor associated lymphocytes and macrophages can enhance tumor cell growth and metastasis by promoting their survival, proliferation, migration to and invasion of other tissues [Beyaert et al., 2013].

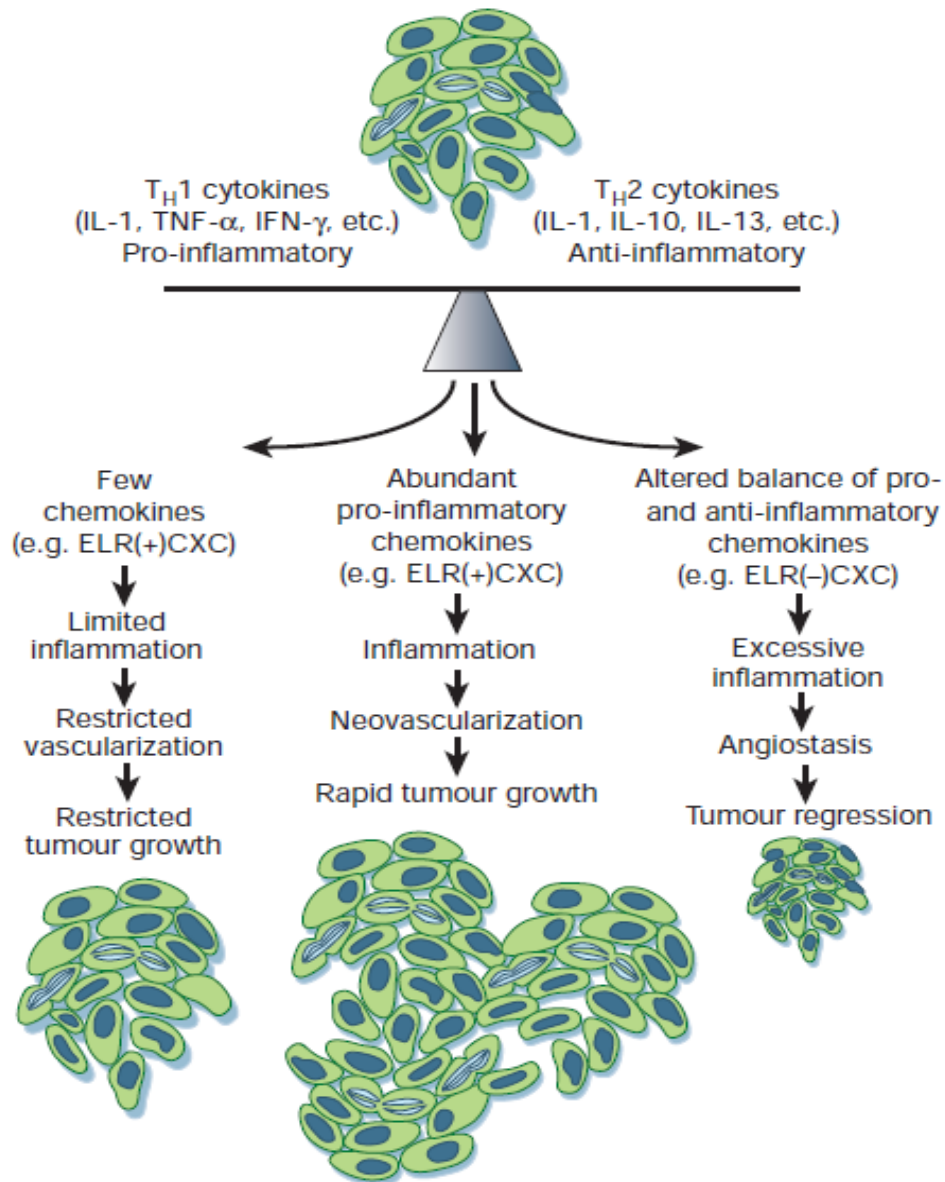


Figure 7.2: Cytokine and chemokine balances regulate neoplastic outcome. [From: Coussens and Werb 2002].

7.1.1.1 Interferon gamma (IFN- γ)

Interferons (IFNs) are proteins that share significant amino acid homology with each other and exhibit similar biological effects on target cells, including antiviral, anti-proliferative, and immunomodulatory activities [Platanias and Fish 1999]. There are two major classes of human interferons: type I and type II [Platanias and Fish 1999; Schroder et al., 2004]. The family of type I IFNs includes three different subtypes, IFN- α , IFN- β and IFN- ω ; while there is only one type II IFN i.e. IFN- γ [Platanias and Fish 1999; Schroder et al., 2004]. The current study will focus on IFN- γ . IFN- γ is a homodimer glycoprotein consisting of 21-24 kDa subunits, produced by T-cells, natural killer (NK) cells, B-cells and professional antigen-presenting cells (APCs) [Kedzierska and Crowe 2001; Schroder et al., 2004].

IFN- γ is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus [Schroder et al., 2004]. It plays a pivotal role in the regulation of the host immune response against viral and intracellular bacterial pathogens [Frucht et al., 2001; Roff et al., 2014]. High concentrations of IFN- γ are secreted by T_H1 cells, CD8⁺ cytotoxic T lymphocytes, and NK-cells during active infection [Roff et al., 2014]. IFN- γ has a major effect on the regulation of antigen presentation by macrophages and dendritic cells, and in induction of class switching of B-cells [Roff et al., 2014]. IFN- γ production by professional APCs acting locally is important in cell self-activation and activation of nearby cells [Schroder et al., 2004]. In addition, IFN- γ is one of the most important endogenous mediators of immunity and inflammation [Hu and Ivashkiv 2009]. IFN- γ plays a key role in macrophage activation, inflammation, host defence against intracellular pathogens,

T_H1 cell responses, and tumor surveillance and immune editing [Hu and Ivashkiv 2009]. In addition, IFN- γ exerts regulatory functions to limit tissue damage associated with inflammation and to modulate T helper and regulatory T-cell differentiation [Hu and Ivashkiv 2009]. IFN- γ primarily signals through the JAK/STAT pathway (figure 7.1), a pathway used by over 50 cytokines, growth factors, and hormones to affect gene regulation [Schroder et al., 2004]. It has been shown that throughout the acute stage of HIV-1 infection, IFN- γ concentrations increase steadily with a peak approximately 20-24 days post infection [Roff et al., 2014].

7.1.1.2 Interleukin-1 beta (IL-1 β)

Interleukin-1 is a large family of cytokines that mediate innate immune responses to defend the host against pathogens [Acuner Ozbabacan et al., 2014]. There are 11 members of the IL-1 family of ligands including IL-1 α , IL-1 β , and IL-1 receptor antagonist [Dinarello 2009; Dinarello 2011]. The first discovered family members are IL-1 α and IL-1 β , and they are secreted by macrophages and epithelial cells in response to pathogens and they have strong pro-inflammatory properties leading to fever and activation of T-cells and macrophages [Acuner Ozbabacan et al., 2014]. IL-1 β is a 17kDa protein encoded on the long arm of chromosome 2 and is produced mainly by monocytes, macrophages, and dendritic cells in response to a variety of bacterial products, principally via interactions with toll-like receptors [Connolly et al., 2005]. In addition, IL-1 β is a pro-inflammatory cytokine produced predominantly by cells of macrophage lineage, and to a lesser extent by B-cells, endothelial cells and fibroblasts in response to infections and inflammation [Kedzierska and Crowe 2001].

IL-1 β is synthesized as an inactive precursor requiring cleavage by IL-1 β converting enzyme, also known as caspase-1 to the active cytokine [Connolly et al., 2005]. IL-1 β can mediate inflammatory responses by supporting T-cell survival, up-regulation of the IL-2 receptor on lymphocytes, enhancing antibody production of B-cells and by promoting B-cell proliferation and T_H17 cell differentiation [Krause et al., 2012]. Apart from its role in host defence, IL-1 β is known to be important in a number of severe inflammatory diseases including the rare cryopyrin-associated periodic syndromes and other hereditary and polygenic auto-inflammatory diseases [Krause et al., 2012]. In humans, blocking IL-1 activity, particularly IL-1 β has entered clinical medicine [Dinarello 2009]. Clinically, elevated IL-1 β concentrations have been associated with many human diseases, and blocking IL-1 β activity is currently the standard therapy for auto-inflammatory diseases [Karimbux et al., 2012].

It has been reported that HIV-1 replication is increased by the presence of IL-1 β , but this effect is complex and appears to be related to both the presence of additional replication stimuli and the specific viral subspecies [Connolly et al., 2005]. In addition, there is conflicting data on the level of expression of IL-1 β in HIV-1 infected individuals. Some studies show decreased concentrations, while others show increased IL-1 β concentrations in progressive HIV disease [Connolly et al., 2005]. It has been shown that IL-1 β contributes to increased *in vitro* susceptibility of both naïve and memory CD4 and CD8 T-cells to apoptosis in the setting of HIV-1 infection [Connolly et al., 2005].

7.1.1.3 Interleukin-2 (IL-2)

Interleukin-2 (IL-2) a 14-17kDa monomeric glycoprotein, is a T_H1 cytokine that is produced by T-cells and it regulates the proliferation and survival of lymphocytes, mainly CD4 cells [Kedzierska and Crowe 2001; Molina et al., 2009; de Lastours et al., 2014]. IL-2 mediates a number of biologic activities that may promote immune system functions [Mitsuyasu 2001]. The primary role of IL-2 is to stimulate the proliferation of activated T-cells, cytotoxic activity of CD8 T-cells and NK-cells, as well as inducing B-cells and monocytes [Kedzierska and Crowe 2001]. During steady-state conditions, IL-2 is mainly produced by CD4 T-cells in secondary lymphoid organs and, to a lesser extent by CD8 T-cells, NK-cells and NKT-cells [Boyman and Sprent 2012]. Under certain conditions, IL-2 can also be synthesized in small amounts by activated dendritic cells (DCs) and mast cells [Boyman and Sprent 2012]. IL-2 production by CD4 and CD8 T-cells is strongly induced following activation by antigen [Boyman and Sprent 2012].

IL-2 production is regulated by several mechanisms, including silencing of IL-2 gene by the transcription factor B lymphocyte-induced maturation protein 1 (BLIMP1) [Boyman and Sprent 2012]. IL-2 acts on cells expressing either the high-affinity IL-2R or the low-affinity dimeric IL-2R [Boyman and Sprent 2012]. IL-2 signaling has been associated with T-reg cell development, homeostasis and function [Fontenot et al., 2005]. IL-2 is crucial for the maintenance of immune homeostasis, as it plays a central role in down-regulating immune responses and its absence results in severe autoimmunity due to a failure to eliminate activated T-cells [Gaffen and Liu 2004]. IL-2 plays a complex immune-regulatory role by inducing activated cells to enter pre-

apoptotic phase, increasing the levels of production of pro-inflammatory cytokines, and influencing T-cell differentiation [De Paoli 2001]. Several functions mediated by IL-2 suggest that it might be useful as an anticancer agent; as it supports the growth of cytotoxic cells, enhances the cytotoxicity of NK-cells, and is essential for the induction of lymphokine-activated killer cells [De Paoli 2001]. In addition, IL-2 therapy has been shown to lead to substantial increases in CD4 T-cell count and decreases the viral load in HIV-1 infected patients [Weissman et al., 2000; Josefson 2000; Sereti et al., 2004; de Lastours et al., 2014].

7.1.1.4 Interleukin-4 (IL-4)

Interleukin-4 (IL-4) an 18kDa monomeric protein, is a T_H2 cytokine produced predominantly by activated CD4 T-cells, NK-cells, mast cells and basophils [Kedzierska and Crowe 2001]. IL-4 is a multifunctional pleiotropic cytokine that participates in the regulation of the immune system at multiple levels [Zamorano et al., 2003; Goldstein et al., 2011; Luzina et al., 2012]. It is the mediator produced mainly by activated T-cells but also by mast cells, basophils, and eosinophils [Luzina et al., 2012]. IL-4 acts upon a broad range of targets, including haematopoietic cells, endothelial cells and tumor cells [Goldstein et al., 2011]. IL-4 is a growth and survival factor for lymphocytes [Zamorano et al., 2003]. It stimulates B-cell activation and differentiation, secretion of IgG1 and IgE, T-cell activation and MHC II expression on B-cells and macrophages [Kedzierska and Crowe 2001]. Although it was discovered as a B-cell differentiation and stimulatory factor, its role in regulating T-cell differentiation is critical during the immune response [Zamorano et al., 2003].

IL-4 plays an essential role by promoting T_H2 cell differentiation while inhibiting T_H1 cell differentiation [Zamorano et al., 2003]. IL-4 is also able to protect lymphoid cells from apoptosis, but it is unable to promote proliferation of small resting lymphocytes without a co-stimulatory signal such as that provided through antigen receptor engagement [Zamorano et al., 2003]. IL-4 is a potent anti-apoptotic cytokine, therefore it is a survival factor for tumor cells, and it can protect tumor cells from apoptosis induced by multiple agents including those used in anti-tumor therapies [Zamorano et al., 2003]. Thus, it is possible that IL-4 participates in resistance to cancer treatments [Zamorano et al., 2003]. In addition, it has been shown that IL-4 is an important regulator of HIV-1, and plays a critical role in the control of viral evolution and accelerated disease progression has been suggested [Valentin et al., 1998].

7.1.1.5 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a 26kDa pleiotropic cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, monocytes, normal haematopoietic cells and lymphocytes in response to viral or bacterial infection [Emilie et al., 1992; Fayad et al., 2001; Kedzierska and Crowe 2001]. It has a wide spectrum of activities including B-cell stimulation, monocyte differentiation and induction of IL-4 producing cells [Kedzierska and Crowe 2001]. IL-6 mediates B-cell terminal differentiation and maturation as well as antibody synthesis by activated B-cells at the mRNA level [Connolly et al., 2005]. Acting synergistically with IL-1 β and TNF- α , IL-6 is involved in T-cell activation, growth, and differentiation [Connolly et al., 2005].

Dysregulation of IL-6 type cytokine signalling contributes to the onset and maintenance of several diseases including cancer [Heinrich et al., 2003]. It has been shown that IL-6 functions as an autocrine factor in tumor cells and can also act as an attractant for the circulating tumor cells [Kamimura et al., 2014]. Many studies have demonstrated that IL-6 expression and secretion are abnormally high in HIV-1 infected individuals [Connolly et al., 2005].

The high levels of IL-6 expression increase both HIV-1 replication in latently infected macrophages and alter the function of infected macrophages, likely rendering them sub-optimally functional [Connolly et al., 2005]. In addition, earlier studies suggested a possible role for dysregulated production of IL-6 in malignant lymphomas [Fayad et al., 2001]. It has been shown that HIV associated B-cell lymphoma cell lines produce large amounts of IL-6 [Fayad et al., 2001]. Serum IL-6 concentrations have been correlated with an increased risk for the development of lymphoma in HIV+ patients and have been associated with adverse prognostic features, and predictive of a poor failure free and overall survival [Fayad et al., 2001].

7.1.1.6 Interleukin-8 (IL-8)

Interleukin-8 (IL-8) is predominantly a 72 amino acid peptide expressed in many cell types and is released in response to pro-inflammatory stimuli [Xiong et al., 2003]. IL-8 was originally identified as a neutrophil chemotactic factor in the supernatants of activated human monocytes [Xie 2001]. IL-8 alternatively known as CXCL8, is a pro-inflammatory CXC α chemokine and its main function is chemotaxis of neutrophils and T-cells [Zeilhofer and Schorr 2000; Waugh and Wilson 2008; Cui et al., 2012]. Chemokines is a family of pro-inflammatory cytokines playing a role in immune system regulation, cell growth, cell development, and inflammation [Cui et al., 2012]. Chemokines play an important role in attracting granulocytes into sites of inflammation [Petering et al., 1999]. In addition, IL-8 is involved in a wide variety of physiologic and pathophysiologic processes ranging from host defence against bacterial infections and phagocytosis of necrotic tissue to numerous autoimmune disorders [Zeilhofer and Schorr 2000].

Furthermore, it has been suggested that IL-8 is produced by various normal and tumorigenic human cells [Xie 2001]. IL-8 is the inflammatory and antimicrobial cytokine produced by macrophages, T-cells, neutrophils and endothelial cells in acute and chronic inflammatory states [Kedzierska and Crowe 2001]. It is a potent chemotactic factor for T-cells, NK-cells, neutrophils and basophils [Kedzierska and Crowe 2001]. IL-8 has also been implicated in many pathological processes involved in cancer progression [Campbell et al., 2013].

7.1.1.7 Interleukin-10 (IL-10)

Interleukin-10 (IL-10) is an 18kDa pleiotropic cytokine produced by T_H2 , as well as monocytes, macrophages and normal and neoplastic B-cells [Fayad et al., 2001; Kedzierska and Crowe 2001]. IL-10 production has strong immunosuppressive effects via inhibition of T_H1 type cytokines, including interferon-gamma and interleukin-2 [Fayad et al., 2001]. It inhibits T-cell proliferation, predominantly by suppressing synthesis of T_H1 cytokines (including IL-2 and FN-gamma), and inhibits macrophage activation and secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, TNF- α) [Kedzierska and Crowe 2001]. In addition, IL-10 has multiple effects on B-cells, including stimulation of growth and differentiation, and thus may contribute to the development of B-cell lymphoma [Blay et al., 1993; Masood et al., 1995]. Increased serum concentrations of IL-10 have been reported in both HIV positive and negative lymphoma patients and such increases have been associated with poor survival [Masood et al., 1995].

7.1.1.8 Interleukin-12p70 (IL-12 p70)

Interleukin-12 (IL-12) is a heterodimer of 70kDa (p70) formed by two covalently linked glycosylated chains of approximately 35kDa (p35) and 40kDa (p40) [Trinchieri 1994; Hauer et al., 2005; Hamza et al., 2010]. IL-12 is produced by various cell types such as monocytes, neutrophils, dendritic cells, and macrophages on activation of these cells by pathogens, by CD40 ligands expressing T-cells, or by extracellular matrix components, such as the glycosaminoglycan hyaluronan [Hauer et al., 2005]. IL-12 has multiple biological functions and importantly, it bridges the early non-

specific innate resistance and the subsequent antigen-specific adaptive immunity [Hamza et al., 2010; Pan et al., 2012]. It is a key factor in the induction of T-cell dependent and independent activation of macrophages, generation of T_H1 and cytotoxic T-cells, suppression of IgG1 and IgE production, induction of organ-specific autoimmunity, and resistance to bacterial and parasitic infections [Ma and Montaner 2000]. IL-12 has been shown to possess potent anti-tumor activity in a wide variety of murine tumor models [Toubai et al., 2006; Pan et al., 2012]. IL-12 induces IFN- γ production by T and NK-cells and thereby contributing to antitumor immunity [Yang et al., 2012; Pan et al., 2012].

Tumor growth depends mainly on the inability of the organism to elicit a potent immune response and on the formation of new blood vessels that enable tumor nutrition [Rodriguez-Galan et al., 2009]. IL-12 therapy can target both processes mainly to its ability to enhance type 1 immunity, to induce IFN- γ expression, and to inhibit tumor angiogenesis mainly through IFN- γ dependent production of the chemokine IP-10 [Rodriguez-Galan et al., 2009]. In addition to activating antitumor effectors, IL-12 and IFN- γ also inhibit the expansion of intra-tumoral T-regs and angiogenesis in the tumor microenvironment, thus enhancing tumor control [Ngiow et al., 2013]. Clinical responses to IL-12 treatment have been reported in many types of tumors, however, systemic administration of IL-12 has been limited by its severe toxic effects [Pan et al., 2012].

7.1.1.9 Interleukin-13 (IL-13)

Interleukin-13 (IL-13) is a predominantly T_H2 derived cytokine that has multiple effects on both haematopoietic and non-haematopoietic cells [Kedzierska and Crowe 2001; Wynn 2003; Mandal 2010]. IL-13 is required for optimal IgE production by B cells, induces MHC II expression on APC and regulates inflammation by inhibiting pro-inflammatory cytokines and chemokine production [Kedzierska and Crowe 2001]. IL-13 is closely related to IL-4 and they display overlapping functions, and the genes for the human protein are both found on chromosome 5q [Jensen 2000]. IL-13 is produced by activated T_H0 , T_H1 like cells, T_H2 like cells and CD8 T-cells [Jensen 2000].

IL-13 has multiple effects on the differentiation and functions of monocytes/macrophages [Jensen 2000]. In addition, it has been reported that IL-13 activates NK-cells and CD8+ T-cells and to induce macrophages to become cells with dendritic cell characteristics [Emilie et al., 1997]. IL-13 exerts its activity via its receptor complex, which consists of the heterodimeric proteins IL-13 α 1 and IL-4R α [Konstantinidis et al., 2008]. It can suppress the cytotoxic functions of monocytes/macrophages, and production of pro-inflammatory cytokines [Jensen 2000].

7.1.1.10 Tumor necrosis factor-alpha (TNF- α)

Tumor necrosis factor-alpha (TNF- α) is a 17kDa protein that belongs to the TNF superfamily of cytokines that comprises of 27 ligands that all share the hallmark extracellular TNF homology domain [Bremer 2013]. These TNF ligands are typically expressed as type II transmembrane proteins and they play a role in diversified roles in the body [Bremer 2013; Aggarwal et al., 2012]. TNF ligands exert their biological function by binding to and activation of members of the TNF receptor superfamily [Bremer 2013]. TNF- α is a pro-inflammatory cytokine forming a homotrimer capable of cross-linking TNF receptors [Kedzierska and Crowe 2001]. TNF- α is produced by a wide variety of cells, including monocytes, macrophages, T-cells, B-cells, NK-cells, neutrophils and microglia cells [Kedzierska and Crowe 2001].

TNF- α , depending on the target cell type, can mature and activate APC, induce IL-1 β , IL-8, GM-CSF, M-CSF, and IFN- γ from monocytes, and induce apoptosis of mature T-cells [Connolly et al., 2005]. TNF- α can also act as a potent inhibitor of IL-12. Pathological overproduction of TNF- α has been implicated in a variety of disease states including autoimmune diseases and cancer [Connolly et al., 2005]. It has been suggested that TNF- α is essential for killing tumor cells and has been studied as therapy for certain types of cancer [Connolly et al., 2005]. It is well established that the expression of TNF- α is greatly increased in HIV-1 infection and that these levels increase with disease progression [Kedzierska and Crowe 2001; Connolly et al., 2005]. HIV-1 induces TNF- α expression, and exogenous TNF- α enhances HIV-1 replication and positive correlation between increased concentrations of TNF- α and increased plasma HIV-1 viral load has been demonstrated [Connolly et al., 2005].

However, the mechanisms by which TNF- α increases HIV-1 replication are incompletely understood. In addition, it has been shown that patients with malignant lymphomas have high circulating concentrations of both cytokines and that higher plasma concentrations of TNF- α are associated with poor disease outcome [Warzocha et al., 1998].

7.2 Specific Aims

The aim of this study was to determine,

- The serum concentrations of circulating inflammatory cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF- α) in HIV+NHL patients and control populations
- Whether cART has an impact on the serum concentrations of these inflammatory cytokines

7.3 Materials and Methods

7.3.1 Materials

V-PLEX pro-inflammatory panel 1 (human) kit pre-coated with IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF- α antibodies (Catalogue number: K15049D-1) were purchased from Meso-scale discovery (MSD) supplier. Serum separator (SST) tubes (Catalogue number: BD367955) were purchased from the Scientific group PTY LTD supplier.

7.3 2 Methods

7.3.2.1 Sample preparation

Blood samples were collected using serum separator tubes (SST) and put on ice immediately. Samples were allowed to clot for 2 hours, and were centrifuged for 20 minutes at 2000xg. Resultant serum was collected and aliquoted into cryo-tubes and was stored at -20°C. Prior to use in the assay, samples were thawed at room temperature and mixed gently. Samples were then diluted 2 fold with diluent 2 and analysed with meso-scale discovery (MSD) assay.

7.3.2.2 Meso-scale discovery (MSD) assay

Meso-scale discovery (MSD) cytokine assays provide a rapid and convenient method for measuring the concentrations of protein targets within a single, small volume of sample [MSD 2014]. The assays in the Pro-inflammatory panel 1 (human) are sandwich immunoassays. MSD plates are coated with capture antibodies on independent and well defined spots in a specific layout. Multiplex assays are provided on 2 or more spots multi spot plates (figure 7.3). When samples are added, analytes in the sample bind to captured antibodies immobilized on the working electrode surface.

A solution containing the detection antibodies conjugated with electrochemiluminescent labels is then added, and the detection antibodies are recruited by the bound analytes to complete the sandwich. MSD buffer that creates the appropriate chemical environment for electrochemiluniscence is then added and the plate is loaded into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample.

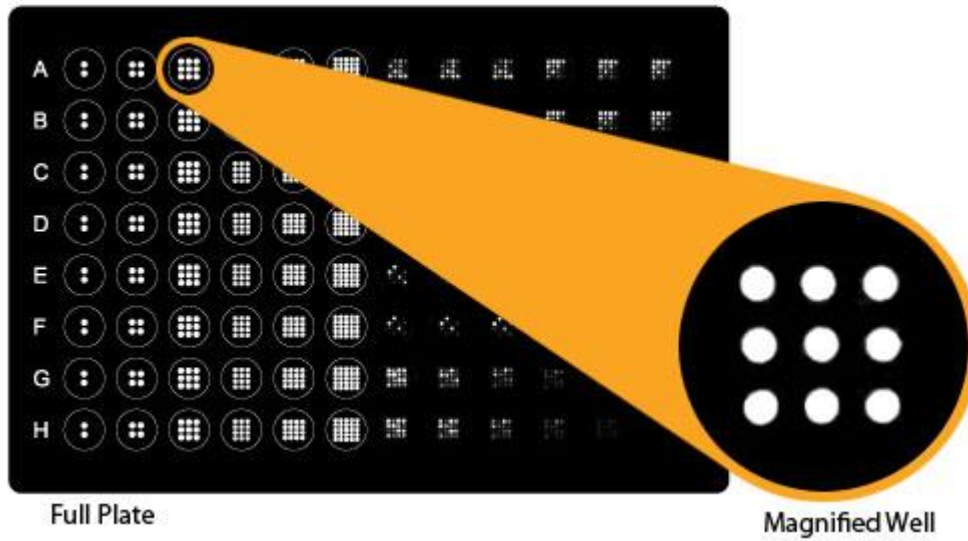


Figure 7.3 *Multiplex Assay Plate*. A full plate consisting of multiple wells with different number of analyte spots (2 and above). Also shown is the magnified single well showing the arrangement spot in a well. [From: Quansys Bioscience 2013]

7.3.2.3 Protocol

In the current study, MSD pro-inflammatory panel 1 (human) kit was used to determine the serum concentrations of IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF- α as follows. Fifty μ l of the diluted samples (section 7.3.2.1), calibrators and controls were added into each well respectively. The plate was sealed with an adhesive plate sealer and incubated at room temperature with shaking for 2 hours. Following the incubation, the plate was washed 3 times with 150 μ l/well of wash buffer. Twenty five μ l of the detection antibody solution was added into each well and the plate was sealed with adhesive plate sealer and incubated at room temperature with shaking for 2 hours. Following the incubation, the plate was washed 3 times with wash buffer. Hundred and fifty μ l of 2x read buffer Twin was added into each well and the plate was read on the MSD instrument.

7.4 Results

7.4.1 Serum concentrations of circulating interferon gamma (IFN- γ)

There was no significant difference in the serum concentrations of circulating IFN- γ when HIV positive NHL (HIV+NHL) patients were compared to HIV negative NHL (NHL) patients (figure 7.4). The serum concentrations of circulating IFN- γ were significantly higher in HIV+NHL patients than HIV positive patients on a cART regimen (HIV+ cART) (26.719 ± 25.632 vs 10.458 ± 8.920 ; $p=0.0085$). NHL patients had significantly higher serum concentrations of circulating IFN- γ than controls (20.425 ± 29.907 vs 5.734 ± 2.328 ; $p=0.0018$). As compared to cART-naïve HIV+ patients, HIV+ cART patients had significantly lower serum concentrations of circulating IFN- γ (10.458 ± 8.920 vs 33.067 ± 9.340 ; $p<0.0001$). cART-naïve HIV+ patients had significantly higher serum concentrations of circulating IFN- γ than controls (33.067 ± 9.340 vs 5.734 ± 2.328 ; $p<0.0001$).

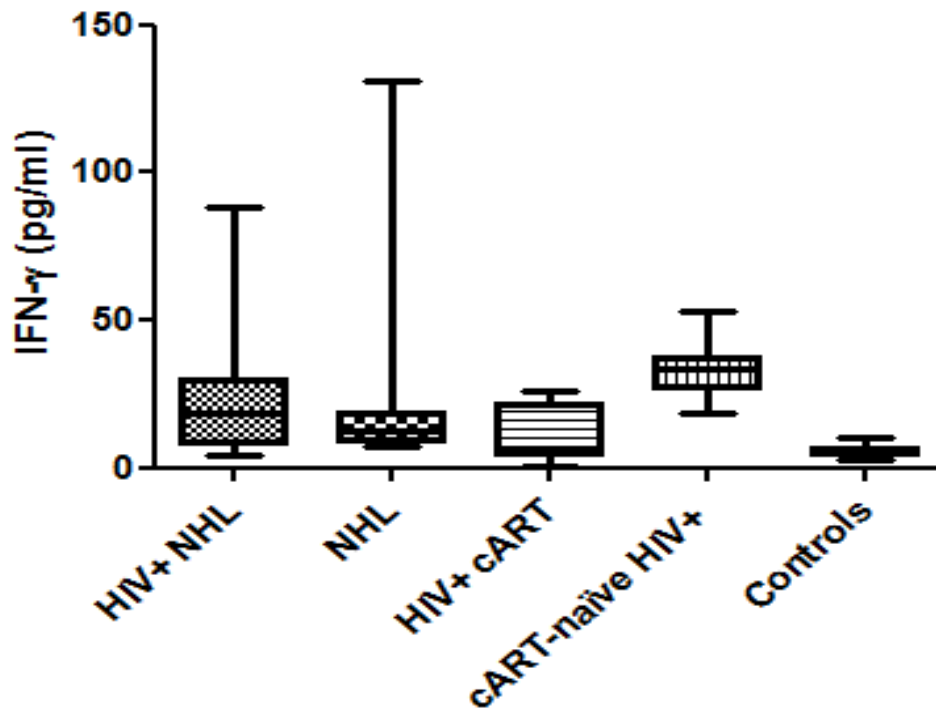


Figure 7.4 Serum concentrations of circulating interferon gamma (*IFN-γ*). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.1.1 Interferon gamma (IFN- γ) correlations

The serum concentrations of circulating IFN- γ were negatively correlated with the numbers of CD4 T-cells ($r=-0.4369$, $p<0.0001$), NKT-cells ($r=-0.2460$, $p=0.0139$), and FoxP3 expression ($r=-0.2891$, $p=0.0047$) (figure 7.5A, D, and F). However, these serum concentrations of circulating IFN- γ were positively associated with the number of CD8 T-cells ($r=0.2200$, $p=0.0250$), NK-cells ($r=0.1903$, $p=0.0454$), and CD8+CD38 expression ($r=0.4919$, $p<0.0001$) (figure 7.5B, C, and E). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association was observed between IFN- γ and HIV+NHL.

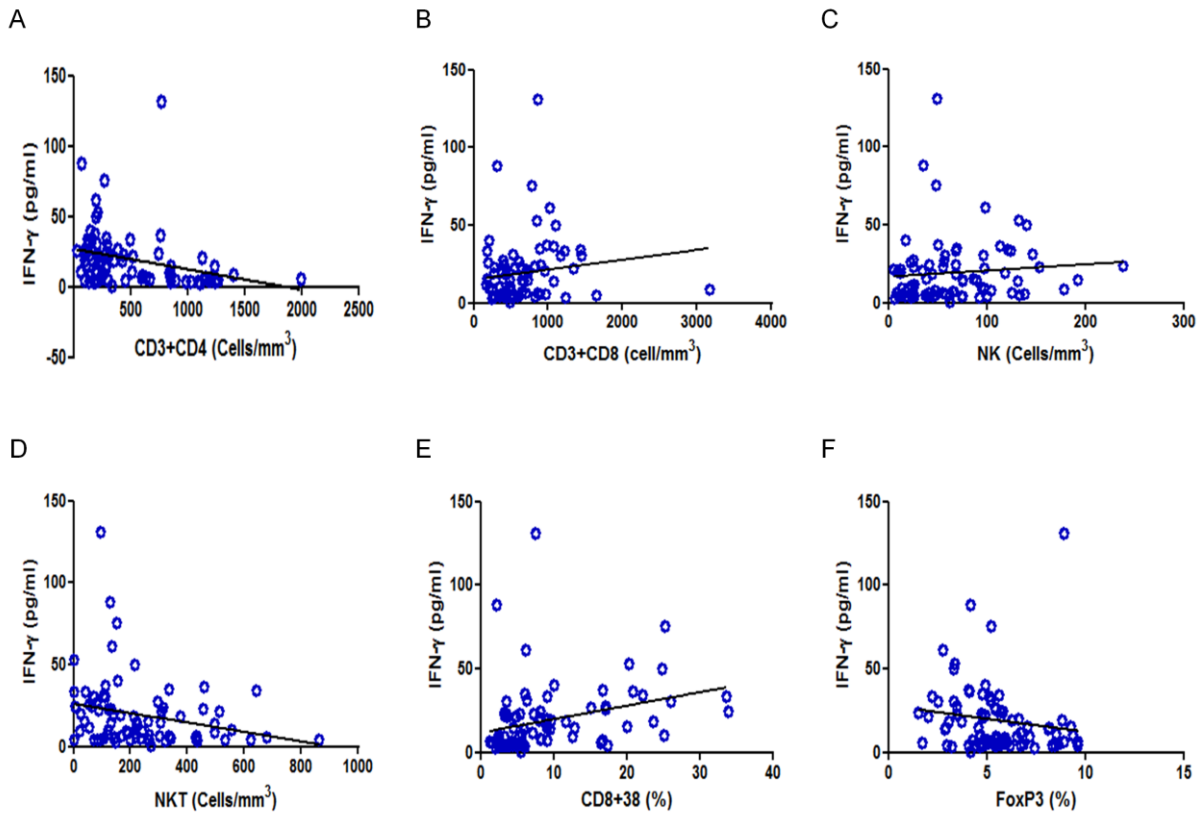


Figure 7.5 Interferon gamma ($IFN-\gamma$) correlations. A) CD3+CD4 vs $IFN-\gamma$ B) CD8 vs $IFN-\gamma$ C) NK-cells vs $IFN-\gamma$ D) NKT vs $IFN-\gamma$ E) CD8+CD38 vs $IFN-\gamma$ F) FoxP3 vs $IFN-\gamma$. NK, Natural killer cells; NKT, Natural killer T-cells; FoxP3, Forkhead box protein 3.

7.4.2 Serum concentrations of circulating interleukin-1 β (IL-1 β)

The serum concentrations of circulating interleukin-1 β were significantly lower in HIV positive NHL (HIV+NHL) patients as compared to HIV negative NHL (NHL) patients (0.117 ± 0.083 vs 0.196 ± 0.096 ; $p=0.0039$) (figure 7.6). However, there was no significant difference in the serum concentrations of circulating IL-1 β when HIV+NHL were compared with HIV positive patients on a cART regimen (HIV+ cART). The serum concentrations of circulating IL-1 β were significantly higher in NHL patients than controls (0.196 ± 0.096 vs 0.102 ± 0.078 ; $p=0.0003$). As compared to cART-naïve HIV+ patients, HIV+ cART patients had significantly lower serum concentrations of circulating IL-1 β (0.113 ± 0.079 vs 0.201 ± 0.180 ; $p=0.0116$). In addition, cART-naïve HIV+ patients had higher serum concentrations of circulating IL-1 β than controls (0.201 ± 0.180 vs 0.102 ± 0.078 ; $p=0.0033$).

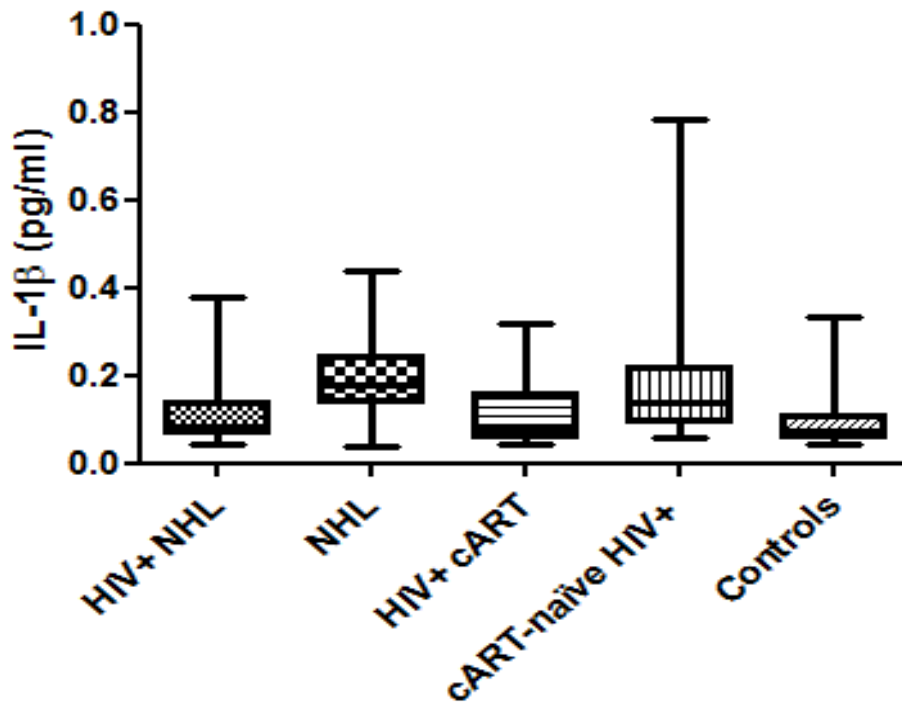


Figure 7.6 Serum concentrations of circulating interleukin-1 β (IL-1 β). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.2.1 Interleukin-1 β (IL-1 β) correlations

The serum concentrations of circulating IL-1 β were positively associated with CD8+38 expression ($r=0.3384$, $p=0.0011$) (figure 7.7A), while they were negatively associated with CD19+ B-cells ($r=-0.2778$, $p=0.0063$) (figure 7.7B). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association between IL-1 β and HIV+NHL was observed.

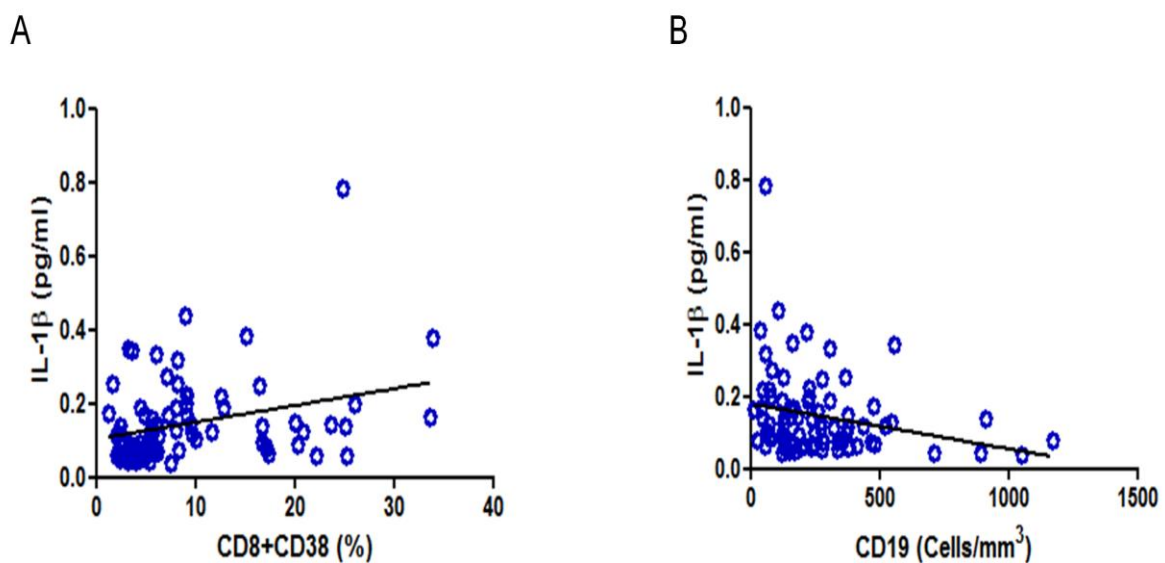


Figure 7.7 Interleukin-1 β (IL-1 β) correlations. A) CD8+CD38 vs IL-1 β B) CD19 vs IL-1 β . IL, Interleukin.

7.4.3 Serum concentrations of circulating interleukin-2 (IL-2)

The serum concentrations of circulating interleukin-2 (IL-2) were significantly higher in HIV positive NHL (HIV+NHL) patients when compared to HIV negative NHL (NHL) patients (0.356 ± 0.135 vs 0.249 ± 0.116 ; $p=0.0115$) (figure 7.8). However, there was no significant difference in the serum concentrations of circulating IL-2 between HIV+NHL and HIV positive patients on a cART regimen (HIV+ cART). NHL patients had significantly higher serum concentrations of circulating IL-2 than controls (0.249 ± 0.116 vs 0.077 ± 0.044 ; $p=0.0013$). The serum concentrations of circulating IL-2 were significantly lower in HIV+ cART than cART-naïve HIV+ patients (0.328 ± 0.148 vs 0.714 ± 0.338 ; $p=0.0038$). cART-naïve HIV+ patients had significantly higher serum concentrations of circulating IL-2 than controls (0.714 ± 0.338 vs 0.077 ± 0.044 ; $p<0.0001$).

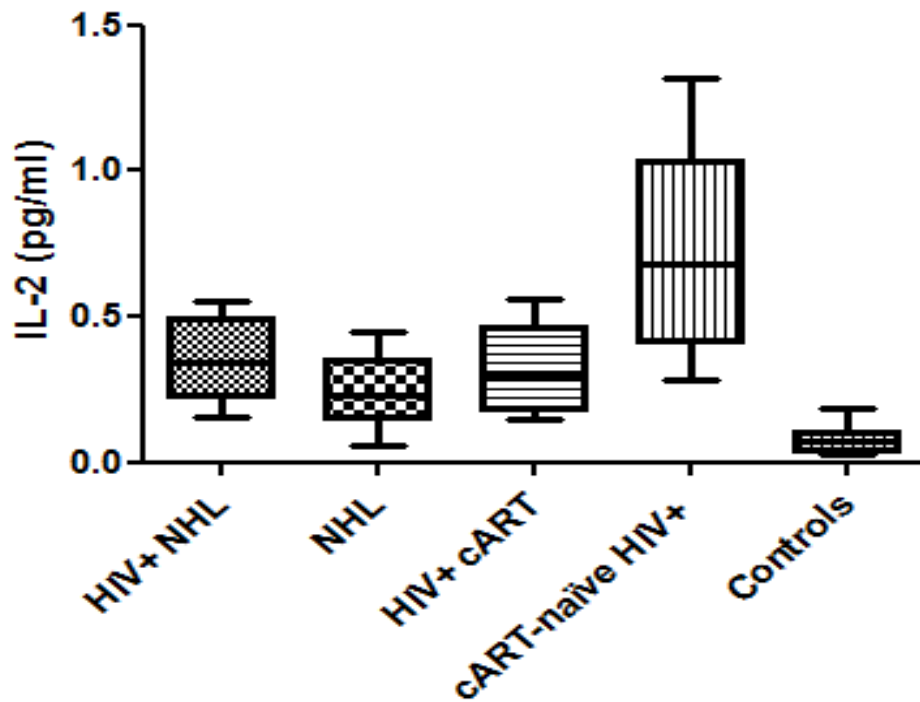


Figure 7.8 Serum concentrations of circulating interleukin-2 (IL-2). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.3.1 Interleukin-2 (IL-2) correlations

The serum concentrations of circulating IL-2 were negatively associated with the numbers of CD4 T-cells ($r=-0.4860$, $p<0.0001$), CD19+ B-cells ($r=-0.3892$, $p=0.0002$), NKT-cells ($r=-0.2315$, $p=0.0194$), and FoxP3 expression ($r=-0.4406$, $p<0.0001$) (figure 7.9A, C, D and F), while they were positively associated with the numbers of CD8 T-cells ($r=0.3135$, $p=0.0023$) and CD8+CD38 expression ($r=0.4014$, $p=0.0001$) (figure 7.9B and E). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association between IL-2 and HIV+NHL was observed.

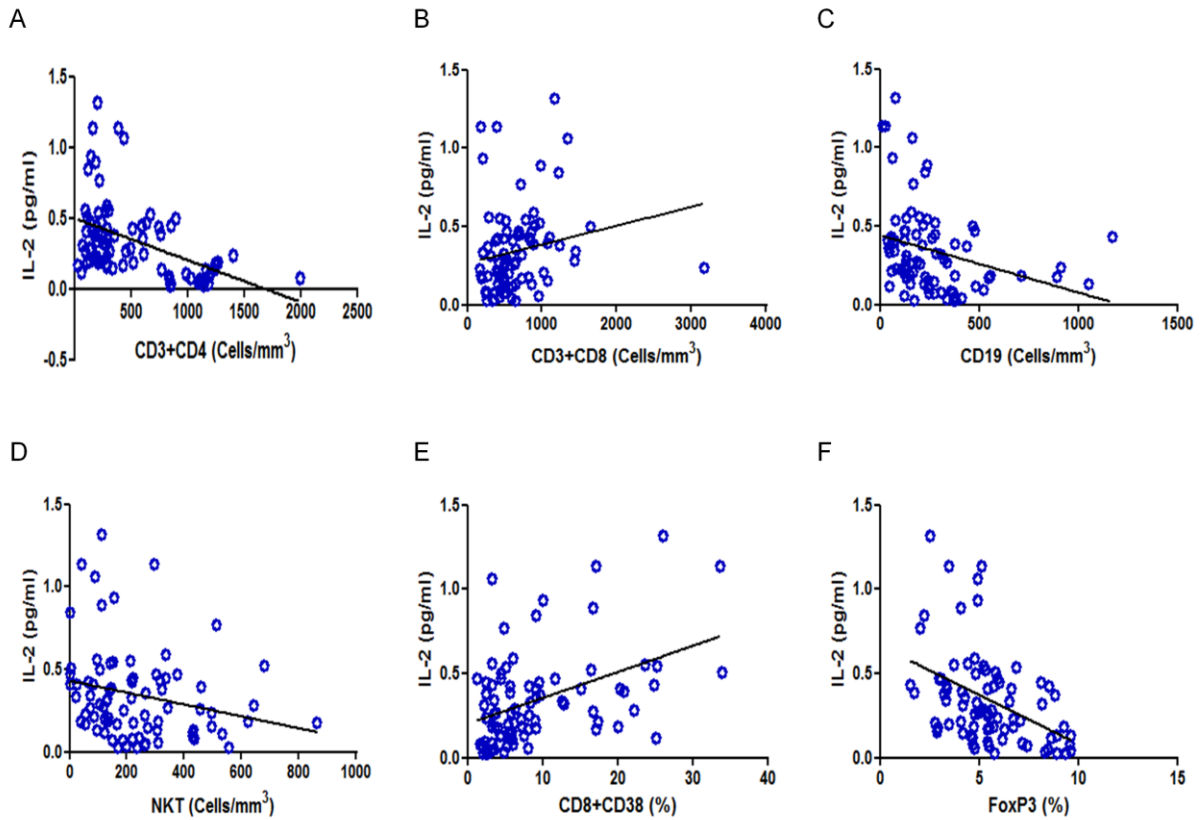


Figure 7.9 Interleukin-2 (IL-2) correlations. A) CD3+CD4 vs IL-2 B) CD3+CD8 vs IL-2 C) CD19 vs IL-2 D) NKT vs IL-2 E) CD8+CD38 vs IL-2 E) FoxP3 vs IL-2. NKT, Natural killer T-cells; FoxP3, Forkhead box protein 3.

7.4.4 Serum concentrations of circulating interleukin-4 (IL-4)

The serum concentrations of circulating interleukin-4 (IL-4) were significantly higher in HIV positive NHL (HIV+NHL) patients as compared to both HIV negative NHL (NHL) patients (0.102 ± 0.036 vs 0.081 ± 0.016 ; $p=0.0234$) and HIV positive patients on a cART regimen (HIV+ cART) (0.102 ± 0.036 vs 0.081 ± 0.026 ; $p=0.03360$) (figure 7.10). As compared to the controls, NHL patients had significantly higher serum concentrations of circulating IL-4 (0.081 ± 0.016 vs 0.056 ± 0.030 ; $p=0.0024$). HIV+ cART patients had significantly lower serum concentrations of circulating IL-4 as compared to cART-naïve HIV+ patients (0.081 ± 0.026 vs 0.112 ± 0.042 ; $p=0.0145$). In addition, cART-naïve HIV+ patients had higher serum concentrations of circulating IL-4 than controls (0.112 ± 0.042 vs 0.056 ± 0.030 ; $p<0.0001$).

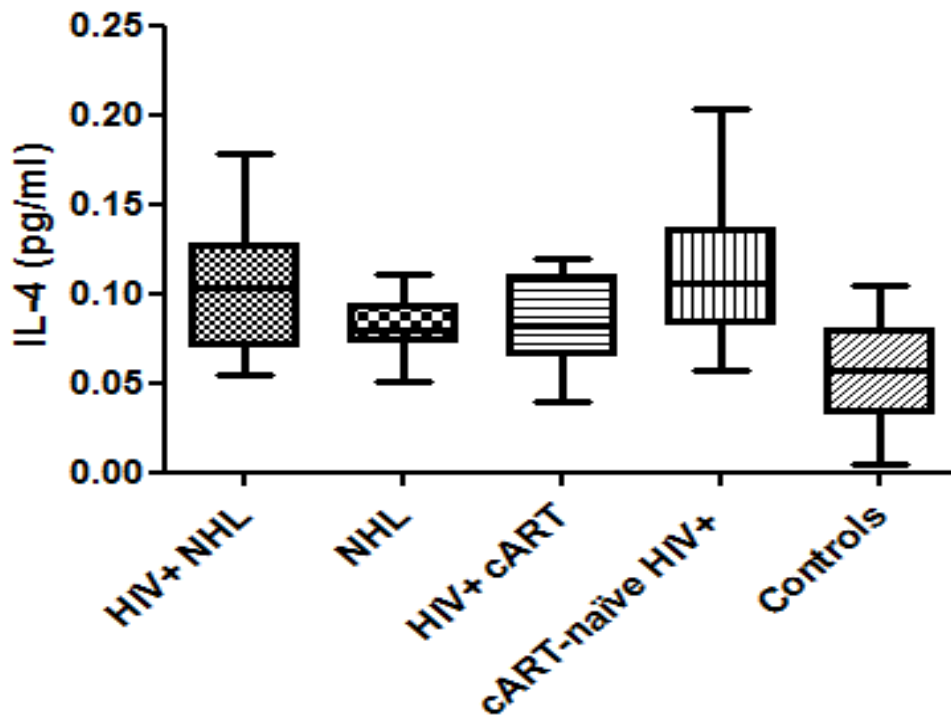


Figure 7.10 Serum concentrations of circulating interleukin-4 (IL-4). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.4.1 Interleukin-4 (IL-4) correlations

The serum concentrations of circulating IL-4 were negatively associated with the numbers of CD4 T-cells ($r=-0.4603$, $p<0.0001$), CD19+ B-cells ($r=-0.3070$, $p=0.0028$), NKT-cells ($r=-0.2249$, $p=0.0224$) and FoxP3 expression ($r=-0.3077$, $p=0.0028$) (figure 7.11A, B, D and F), while they were positively associated with the numbers of NK-cells ($r=0.2853$, $p=0.0052$), and CD8+CD38 expression ($r=0.3220$, $p=0.0018$) (figure 7.11C and E). Following adjustment for age, gender, smoking status, viral load and duration of treatment, the serum concentrations of IL-4 were independently associated with HIV+NHL (OR = 1.26; 95% CI = 0.15–10.18).

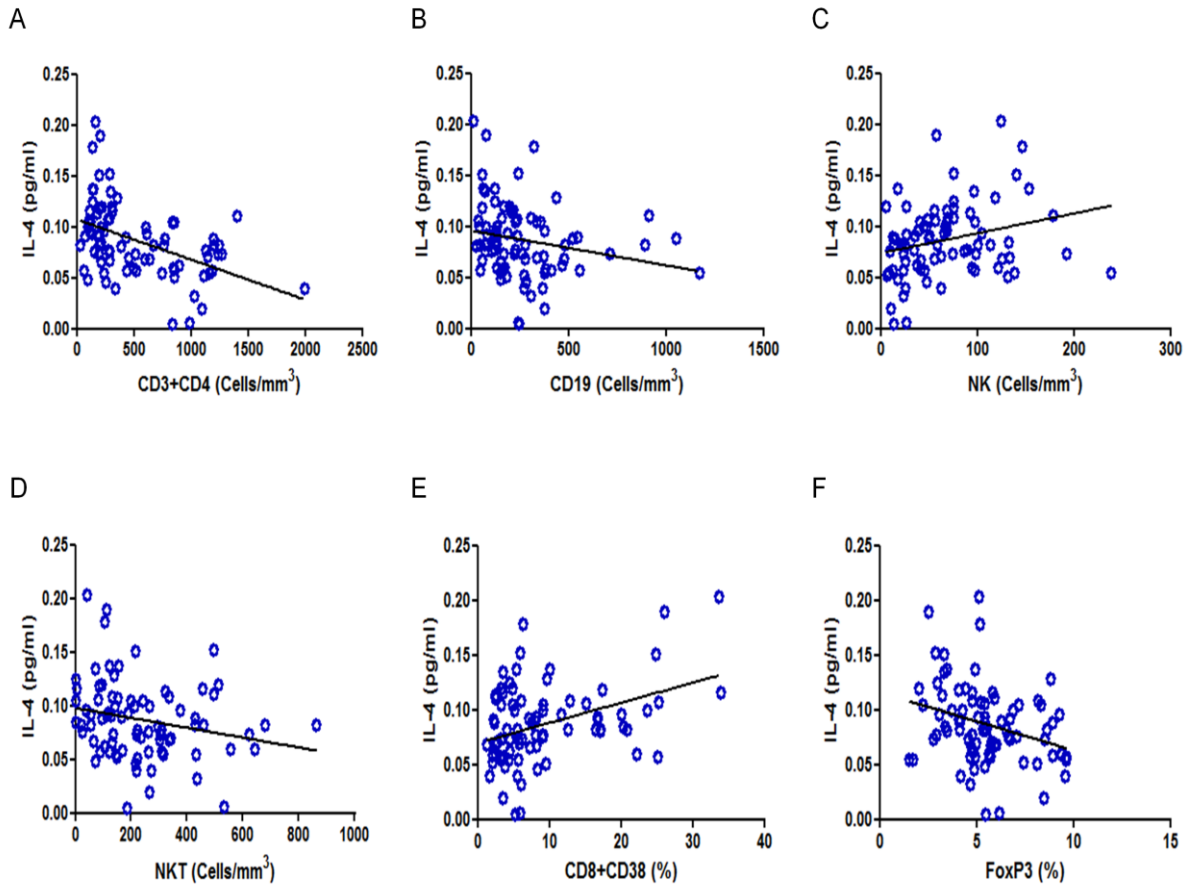


Figure 7.11 *Interleukin-4 (IL-4) correlations.* A) CD3+CD4 vs IL-4 B) CD19 vs IL-4 C) NK vs IL-4 D) NKT vs IL-4 E) CD8+CD38 vs IL-4 F) FoxP3 vs IL-4 . NK, Natural killer; NKT, Natural killer T-cells; FoxP3, Forkhead box protein 3.

7.4.5 Serum concentrations of circulating interleukin-6 (IL-6)

There was no significant difference in the serum concentrations of circulating interleukin-6 (IL-6) between HIV positive NHL (HIV+ NHL) patients and HIV negative NHL (NHL) patients (figure 7.12). The serum concentrations of circulating IL-6 were significantly higher in HIV+NHL patients when compared to HIV positive patients on a cART regimen (HIV+ cART) (1.473 ± 1.256 vs 0.779 ± 0.268 ; $p=0.0265$). NHL patients had higher serum concentrations of circulating IL-6 than controls (1.179 ± 1.171 vs 0.415 ± 0.190 ; $p=0.0013$). The serum concentrations of circulating IL-6 were significantly lower in HIV+ cART as compared to cART-naïve HIV+ patients (0.779 ± 0.268 vs 2.447 ± 1.350 ; $p=0.0003$). In addition, cART-naïve HIV+ patients had significantly higher serum concentrations of circulating IL-6 than controls (2.447 ± 1.350 vs 0.415 ± 0.190 ; $p<0.0001$).

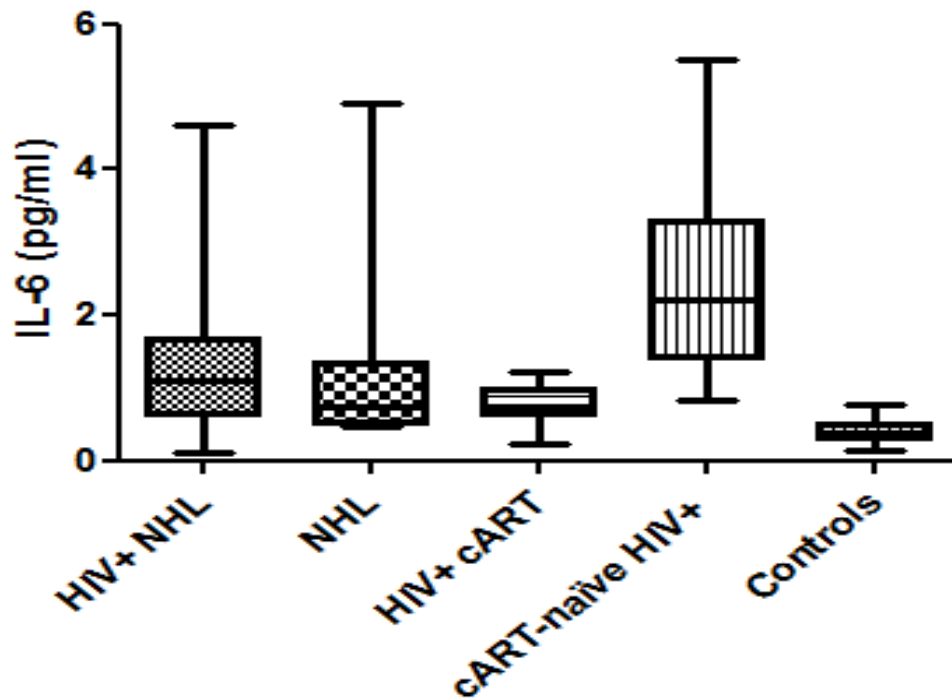


Figure 7.12 Serum concentrations of circulating interleukin-6 (IL-6). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.5.1 Interleukin-6 (IL-6) correlations

The serum concentrations of circulating IL-6 were negatively associated with the numbers of CD4 T-cells ($r=-0.5432$, $p<0.0001$), CD19+ B-cells ($r=-0.4544$, $p<0.0001$), NKT-cells ($r=-0.2616$, $p=0.0095$), and FoxP3 expression ($r=-0.399$, $p=0.0001$) (figure 7.13A, B, C and E), while they were positively associated with CD8+38 expression ($r=0.4139$, $p<0.0001$) (figure 7.13D). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association was observed between IL-6 and HIV+NHL.

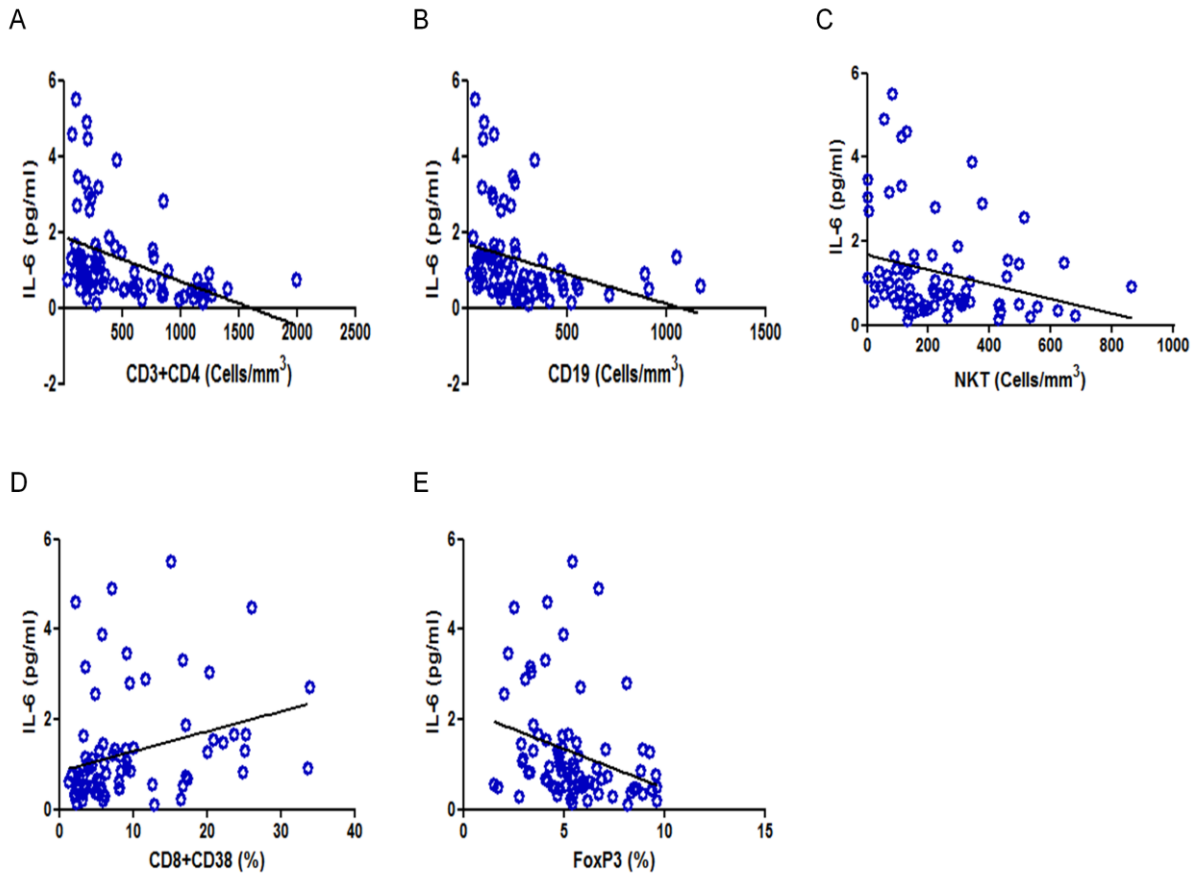


Figure 7.13 Interleukin-6 (*IL-6*) correlations. A) CD3+CD4 vs IL-6 B) CD19 vs IL-6 C) NKT vs IL-6 D) CD8+CD38 vs IL-6 E) FoxP3 vs IL-6. NKT, Natural killer T-cells; FoxP3, Forkhead box protein 3

7.4.6 Serum concentrations of circulating interleukin-8 (IL-8)

There was no significant difference in the serum concentrations of circulating interleukin-8 (IL-8) when HIV positive NHL (HIV+NHL) and HIV negative NHL (NHL) patients were compared (figure 7.14). HIV+NHL patients had significantly higher serum concentrations of circulating IL-8 than HIV positive patients on a cART regimen (HIV+ cART) (13.027 ± 5.341 vs 9.615 ± 5.483 ; $p=0.0219$). The serum concentrations of circulating IL-8 were significantly up-regulated in NHL patients as compared to controls (13.942 ± 7.602 vs 9.110 ± 2.733 ; $p=0.0186$). HIV+ cART patients had significantly lower serum concentrations of circulating IL-8 as compared to cART-naïve HIV+ patients (9.615 ± 5.483 vs 14.002 ± 3.649 ; $p=0.0015$). CART-naïve HIV+ patients had significantly higher serum concentrations of circulating IL-8 than controls (14.002 ± 3.649 vs 9.110 ± 2.733 ; $p=0.0012$).

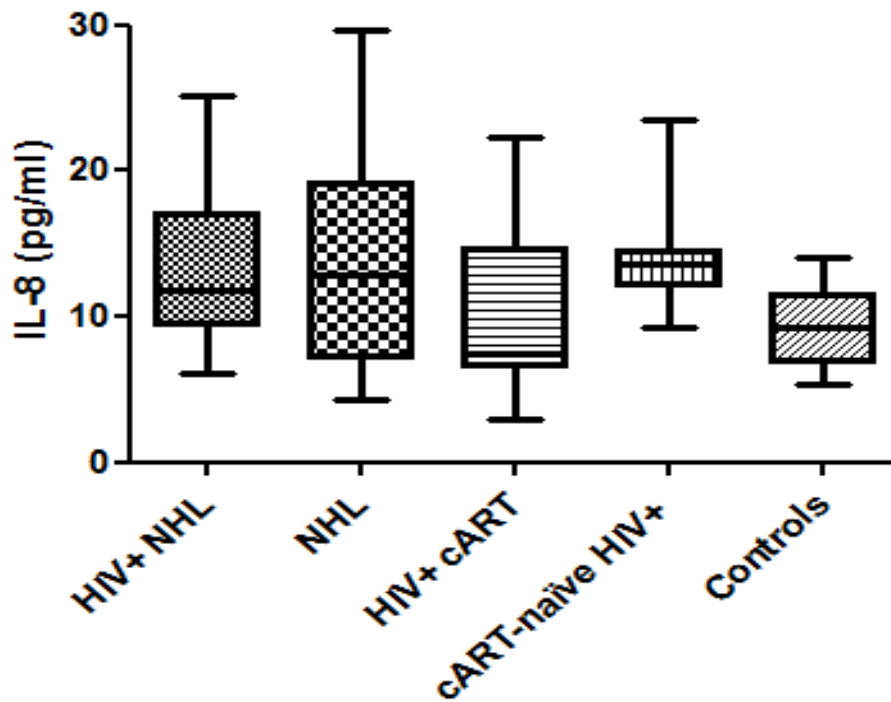


Figure 7.14 Serum concentrations of circulating interleukin-8 (IL-8). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.6.1 Interleukin-8 (IL-8) correlations

The serum concentrations of circulating IL-8 were negatively associated with the numbers of CD4 T-cells ($r=-0.3819$, $p=0.0002$), CD19+ B-cells ($r=-0.4411$, $p<0.0001$) and NKT-cells ($r=-0.3472$, $p=0.0008$) (figure 7.15A, B, C), while they were positively associated with CD8+CD38 expression ($r=0.4357$, $p<0.0001$) (figure 7.15D). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association was observed between IL-8 and HIV+NHL.

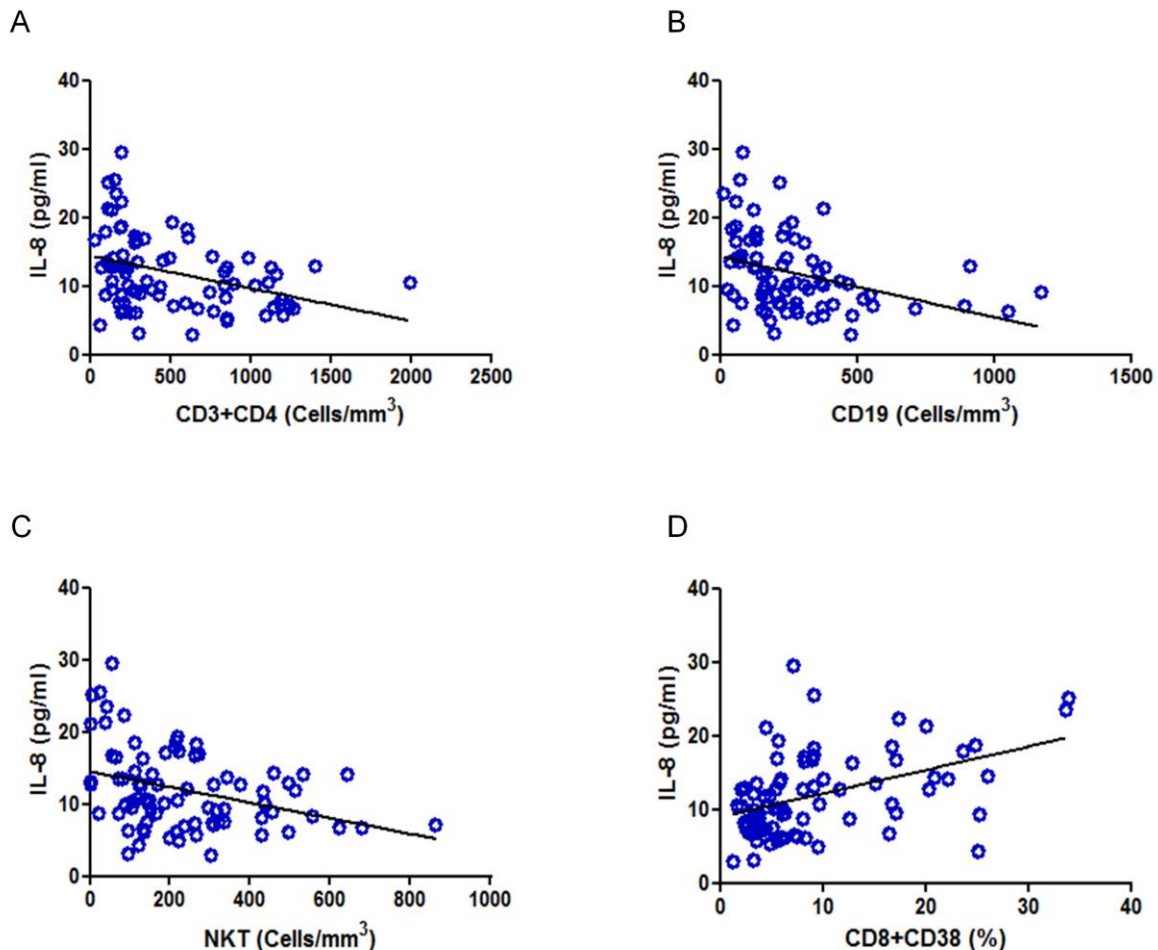


Figure 7.15 Interleukin-8 (IL-8) correlations. A) CD3+CD4 vs IL-8 B) CD19 vs IL-8 C) NKT vs IL-8 D) CD8+CD38 vs IL-8. NKT, Natural killer T-cells.

7.4.7 Serum concentrations of circulating interleukin-10 (IL-10)

There was no significant difference in the serum concentrations of circulating interleukin-10 (IL-10) between HIV positive NHL (HIV+NHL) and HIV negative NHL (NHL) patients (figure 7.16). The serum concentrations of circulating IL-10 were significantly higher in HIV+NHL than HIV positive patients on a cART regimen (HIV+ cART) (1.119 ± 0.691 vs 0.482 ± 0.210 ; $p=0.0044$). NHL patients had significantly higher serum concentrations of circulating IL-10 than controls (1.344 ± 1.550 vs 0.375 ± 0.338 ; $p=0.0003$). HIV+ cART patients had significantly lower serum concentrations of circulating IL-10 than cART-naïve HIV+ patients (0.482 ± 0.210 vs 1.312 ± 0.569 ; $p=0.0002$). The serum concentrations of circulating IL-10 were significantly higher in cART-naïve HIV+ patients as compared to controls (1.312 ± 0.569 vs 0.375 ± 0.338 ; $p<0.0001$).

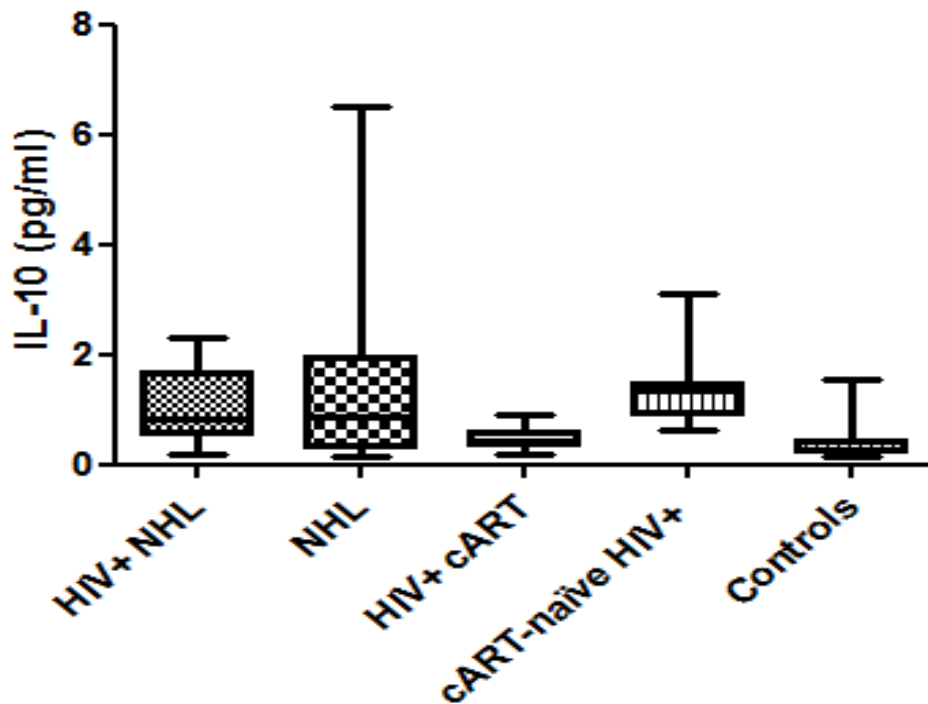


Figure 7.16 Serum concentrations of circulating interleukin-10 (IL-10). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.7.1 Interleukin-10 (IL-10) correlations

The serum concentrations of circulating IL-10 were positively associated with the numbers of NK-cells ($r=0.2086$, $p=0.0317$) and CD8+38 expression ($r=0.3521$, $p=0.0007$) (figure 7.17A and B). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association was observed between IL-10 and HIV+NHL.

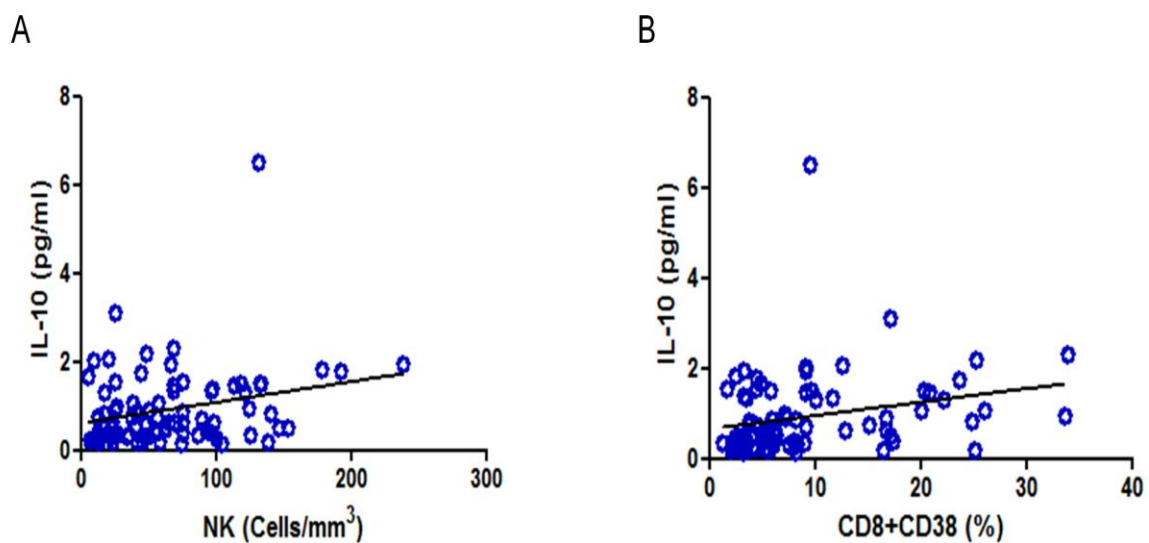


Figure 7.17 Interleukin-10 (IL-10) correlations. A) NK-cells vs IL-10 B) CD8+CD38 vs IL-10. IL, Interleukin; NK, Natural killer cells.

7.4.8 Serum concentrations of circulating interleukin-12p70 (IL-12p70)

There was no significant difference in the serum concentrations of circulating interleukin-12p70 (IL-12p70) when HIV positive NHL (HIV+NHL) and HIV negative NHL (NHL) patients were compared (figure 7.18). HIV+NHL patients had significantly lower serum concentrations of circulating IL-12p70 than HIV positive patients on a cART regimen (HIV+ cART) (0.198 ± 0.115 vs 0.459 ± 0.226 ; $p=0.0012$). The serum concentrations of circulating IL-12p70 were significantly higher in NHL patients as compared to controls (0.250 ± 0.143 vs 0.101 ± 0.091 ; $p=0.0037$). There was no significant difference in the serum concentrations of circulating IL-12p70 between HIV+ cART and cART-naïve HIV+ patients. cART-naïve HIV+ patients had significantly higher serum concentrations of circulating IL-12p70 than controls (0.576 ± 0.245 vs 0.101 ± 0.091 ; $p<0.0001$).

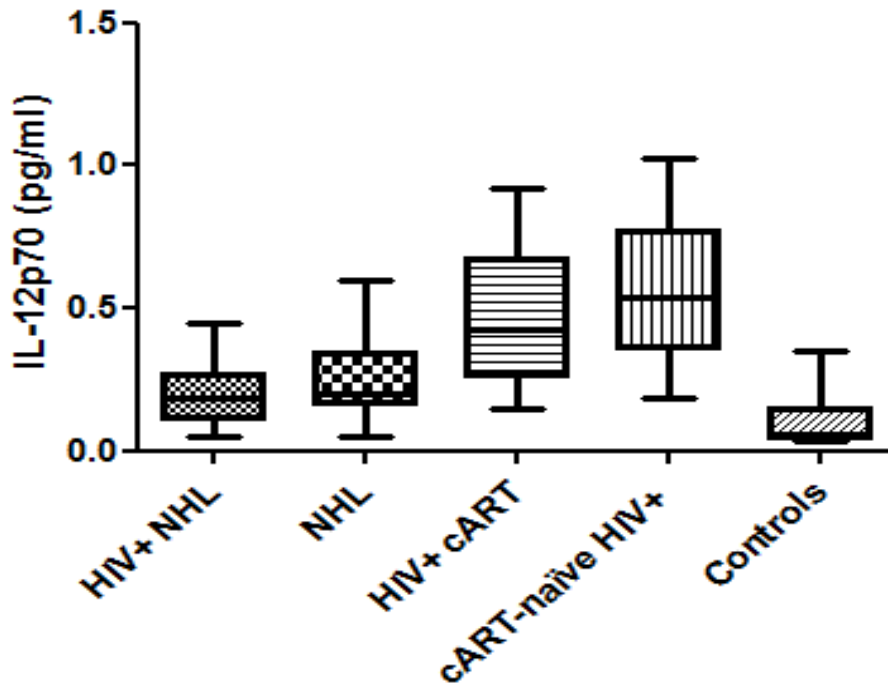


Figure 7.18 Serum concentrations of circulating interleukin-12p70 (IL-12p70). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.8.1 Interleukin-12p70 (IL-12p70) correlations

The serum concentrations of circulating IL-12p70 were negatively associated with the numbers of CD4 T-cells ($r=-0.3439$, $p=0.0009$), CD19+ B-cells ($r=-0.4269$, $p<0.0001$), and FoxP3 expression ($r=-0.3615$, $p=0.0005$) (figure 7.19A, C, and F), while they were positively associated with the numbers of CD8 T-cells ($r=0.2449$, $p=0.0143$), NK-cells ($r=0.195$, $p=0.0415$), and CD8+CD38 expression ($r=0.2245$, $p=0.0227$) (figure 7.19B, D, and E). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association was observed between the serum concentrations of IL-12p70 and HIV+NHL.

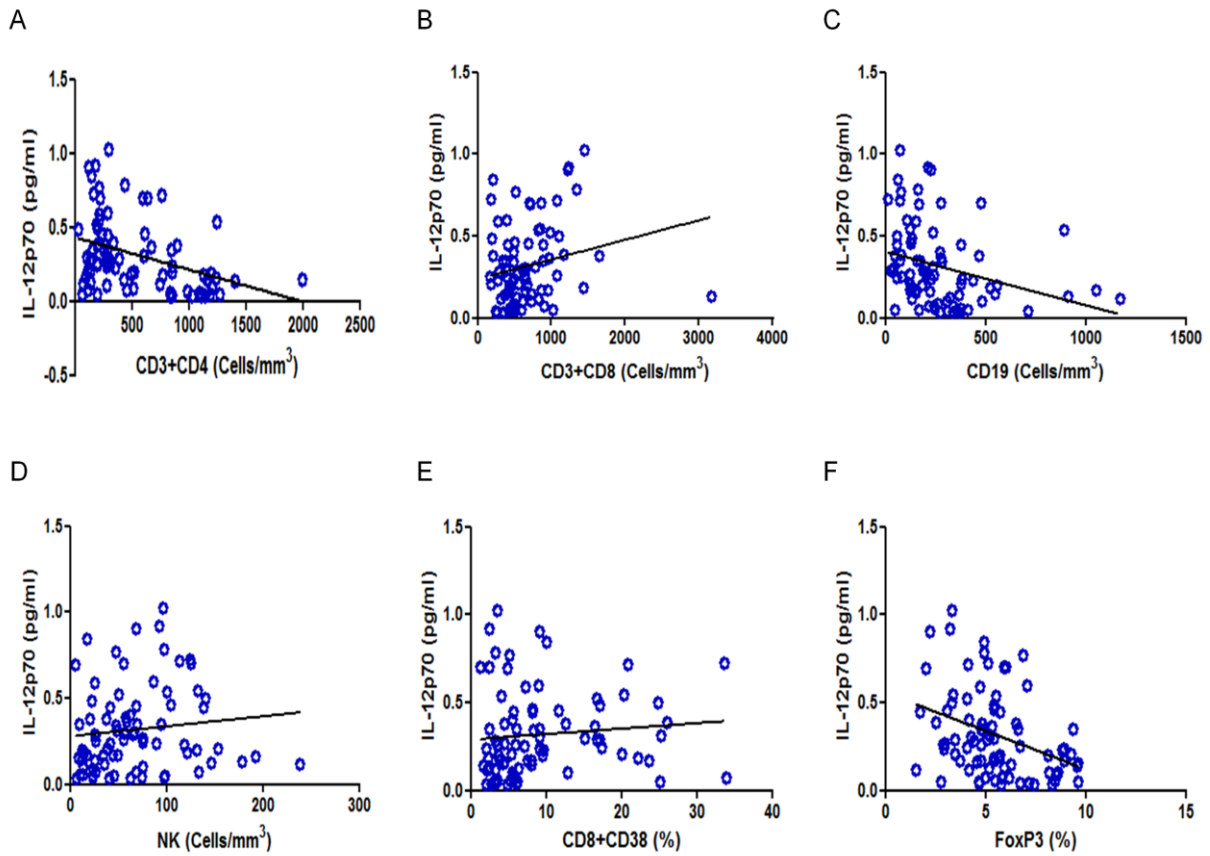


Figure 7.19 Interleukin-12p70 (IL-12p70) correlations. A) CD3+CD4 vs IL-12p70 B) CD3+CD8 vs IL-12p70 C) CD19 vs IL-12p70 D) NK vs IL-12p70 E) CD8+CD38 vs IL-12p70 F) FoxP3 vs IL-12p70. NK, Natural killer cells; FoxP3, Forkhead box protein 3.

7.4.9 Serum concentrations of circulating interleukin-13 (IL-13)

There was no significant difference in the serum concentrations of circulating interleukin-13 (IL-13) when HIV positive NHL (HIV+NHL) and HIV negative NHL (NHL) patients were compared, as well as between HIV+NHL and HIV positive patients on a cART regimen (HIV+ cART) (figure 7.20). NHL patients had significantly higher serum concentrations of circulating IL-13 than controls (0.776 ± 0.249 vs 0.563 ± 0.358 ; $p=0.0317$). There was no significant difference in the serum concentrations of circulating IL-13 between HIV+ cART and cART-naïve HIV+ patients. The serum concentrations of circulating IL-13 were significantly higher in cART-naïve HIV+ patients as compared to controls (0.955 ± 0.285 vs 0.563 ± 0.358 ; $p=0.0013$).

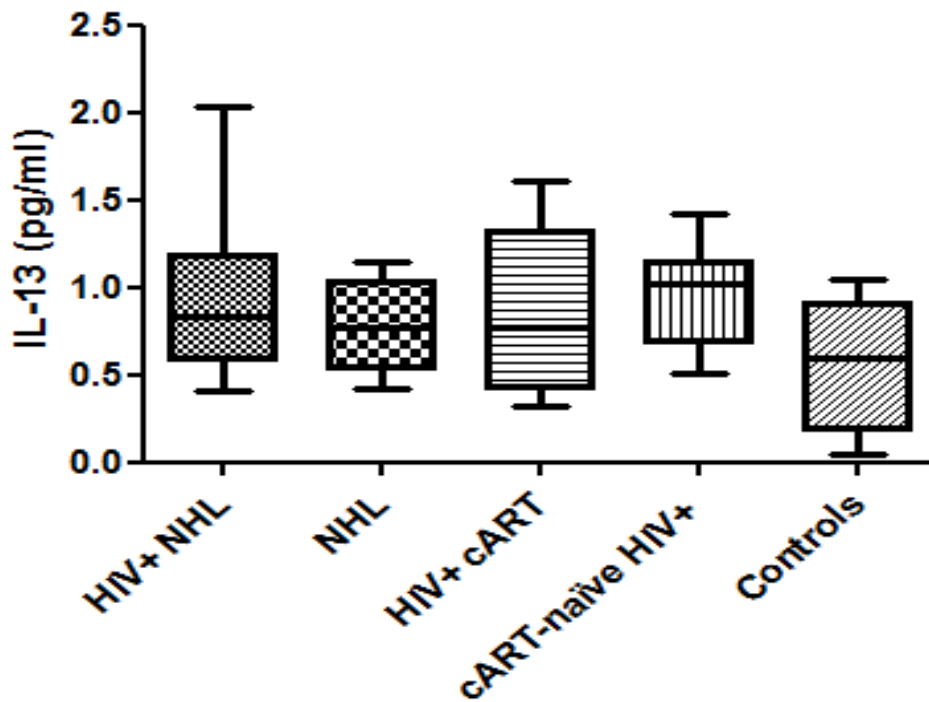


Figure 7.20 Serum concentrations of circulating interleukin-13 (IL-13). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.9.1 Interleukin-13 (IL-13) correlations

The serum concentrations of circulating IL-13 were negatively associated with the numbers of CD4 T-cells ($r=-0.2624$, $p=0.0094$) (figure 7.21A), while they were positively associated with CD8+CD38 expression ($r=0.3588$, $p=0.0005$) (figure 7.21B). Following adjustment for age, gender, smoking status, viral load and duration of treatment, no significant association was observed between IL-13 and HIV+NHL.

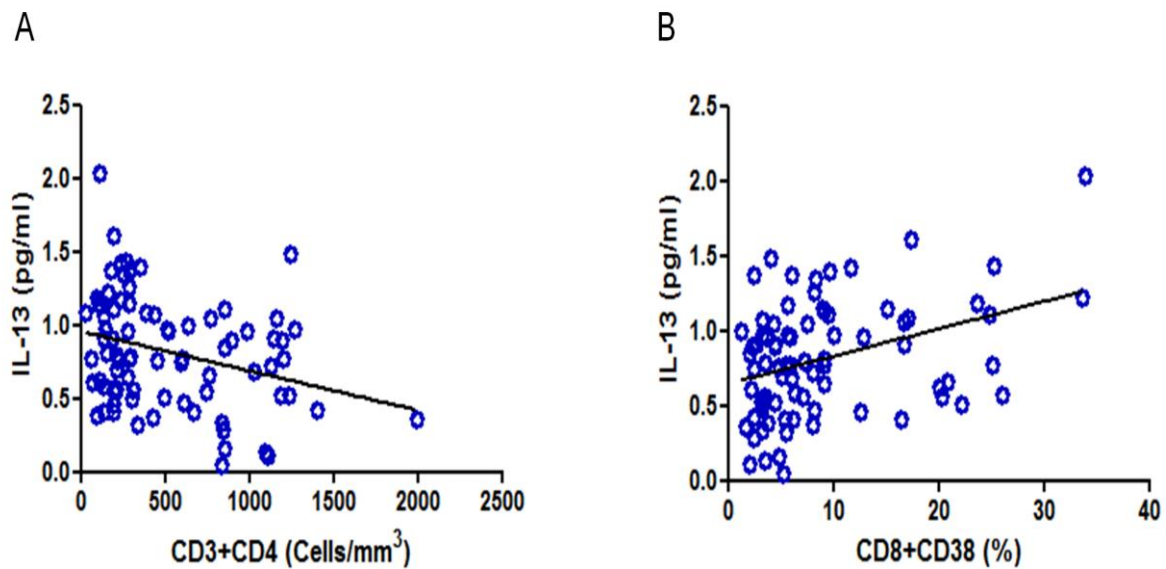


Figure 7.21 Interleukin-13 (IL-13) correlations. A) CD4 vs IL-13 B) CD8+CD38 vs IL-13.

7.4.10 Serum concentrations of circulating tumor necrosis factor- α (TNF- α)

The serum concentrations of circulating tumor necrosis factor- α (TNF- α) were significantly higher in HIV positive NHL (HIV+NHL) patients as compared to HIV negative NHL (NHL) patients (2.012 ± 1.581 vs 1.144 ± 0.394 ; $p=0.0258$) (figure 7.22). However, there was no significant difference in the serum concentrations of TNF- α between HIV+NHL patients and HIV positive patients on a cART regimen (HIV+ cART). As compared to the controls, NHL patients had significantly higher serum concentrations of circulating TNF- α (1.144 ± 0.394 vs 0.925 ± 0.268 ; $p=0.0311$). The serum concentrations of circulating TNF- α were significantly lower in HIV+ cART patients than cART-naïve HIV+ patients (1.342 ± 0.465 vs 3.198 ± 2.029 ; $p=0.0006$). In addition, cART-naïve HIV+ patients had significantly higher serum concentrations of circulating TNF- α than controls (3.198 ± 2.029 vs 0.925 ± 0.268 ; $p<0.0001$).

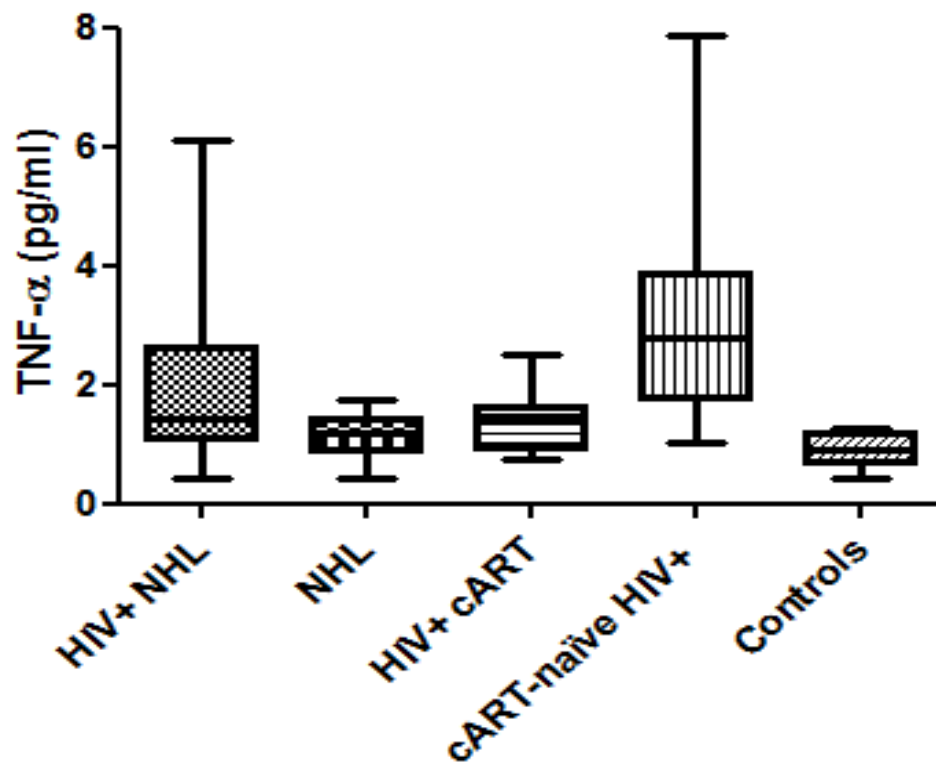


Figure 7.22 Serum concentrations of circulating tumor necrosis factor- α (TNF- α). HIV+NHL, HIV positive non-Hodgkin lymphoma patients (n=31); NHL, HIV negative non-Hodgkin lymphoma patients (n=34); HIV+ cART, combination antiretroviral therapy treated (cART) HIV positive individuals (n=32); cART-naïve HIV+, cART-naïve HIV positive individuals (n=28); Controls, Healthy controls (n=16).

7.4.10.1 Tumor necrosis factor- α (TNF- α) correlations

The serum concentrations of circulating tumor necrosis factor- α (TNF- α) were negatively associated with the number of CD4 T-cells ($r=-0.4508$, $p<0.0001$), CD19+ B-cells ($r=-0.3782$, $p=0.0003$), and FoxP3 expression ($r=-0.4322$, $p<0.0001$) (figure 7.23A, D, and F), while they were positively correlated with the numbers of CD8 T-cells ($r=0.2243$, $p=0.0227$), NK-cells ($r=0.2101$, $p=0.0307$) and CD8+CD38 expression ($r=0.2661$, $p=0.0085$) (figure 7.23B, C and E). Following adjustment for age, gender, smoking status, viral load and duration of treatment, the serum concentrations of TNF- α were independently associated with HIV+NHL (OR = 7.9; 95% CI = 1.17–53.34).

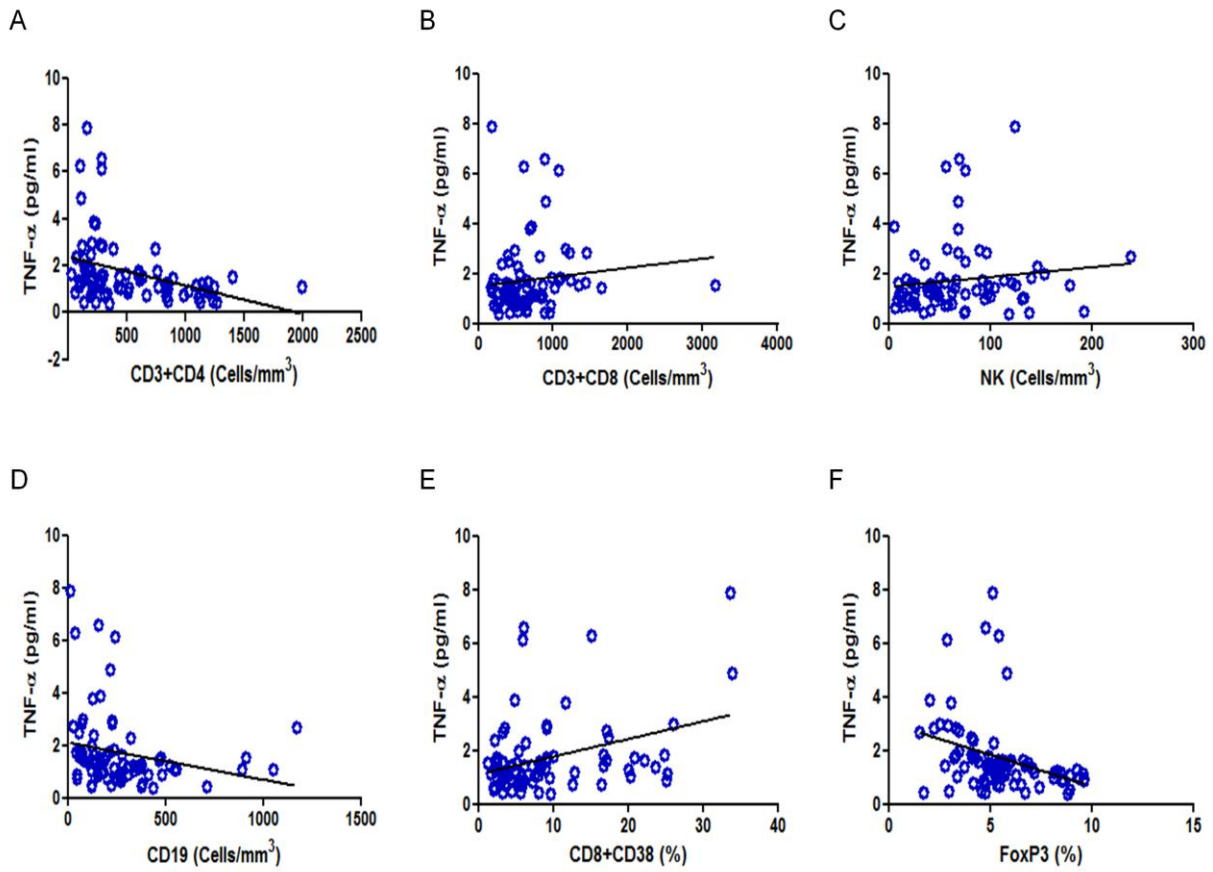


Figure 7.23 *Tumor necrosis factor- α (TNF- α) correlations.* A) CD3+CD4 vs TNF- α B) CD3+CD8 vs TNF- α C) NK vs TNF- α D) CD19 vs TNF- α E) CD8+CD38 vs TNF- α F) FoxP3 vs TNF- α . NK, Natural killer cells; FoxP3, Forkhead box protein 3.

7.5 Discussion

7.5.1 Interferon gamma (IFN- γ)

IFN- γ is known to have a major effect on the regulation of antigen presentation by macrophages, dendritic cells, and in induction of class switching of B-cells [Frucht et al., 2001; Hu and Ivashkiv 2009]. IFN- γ production is detected as early as the acute phase in HIV-1 infected patients, and is known to play various roles in the pathogenesis of HIV disease [Roff et al., 2014]. In the current study, the serum concentrations of circulating IFN- γ were significantly increased in HIV+NHL as compared to HIV+ cART patients. However, there was no significant difference in the serum concentrations of circulating IFN- γ when HIV+NHL patients were compared to NHL patients (figure 7.4). Although there was no significant difference in the serum concentrations of circulating IFN- γ between HIV+NHL and NHL, there was a trend towards increased serum concentrations in HIV+NHL. In addition, NHL had high serum concentrations of circulating IFN- γ when compared to the control population.

IFN- γ plays an important role in tumor protection and rejection [Bax et al., 2013], thus the observed increased serum concentrations of circulating IFN- γ in NHL may have been due to the immune system trying to eradicate malignant lymphoma cells. Gergely and colleagues [2004], reported that IFN- γ production by peripheral T-cell subsets is increased in B-cell NHL patients and this may contribute to strong polarization towards T_H1 type response necessary for lymphoma clearance and remission. The serum concentrations of circulating IFN- γ were significantly elevated in cART-naïve HIV+ patients as compared to HIV+ cART patients as well as when compared to the controls (figure 7.4). These findings suggest that HIV-1 infection

increases the serum concentrations of circulating IFN- γ , and cART reduces them. This may not be a direct effect of cART to the serum concentrations of circulating IFN- γ , but rather an indirect effect of decreasing the viral load. In addition, the serum concentrations of circulating IFN- γ were negatively associated with the numbers of CD4 T-cells, NKT-cells and FoxP3 expression (figure 7.5A, D and F), while they were positively associated with the numbers of CD8 T-cells, NK-cells and CD8+CD38 expression (figure 7.5B, C and E). These results suggest that the decreased immune regulation may lead to increased IFN- γ expression and immune activation.

IFN- γ is initially produced to clear the primary HIV-1 infection, however, the increased serum concentrations observed in the current study may contribute in establishing a chronic immune activation that exacerbates HIV+NHL [Roff et al., 2014]. This is reflected in the increased CD8+CD38 expression, reduced FoxP3 and CD4 T-cell counts in this patient population group. The increased immune activation is a hallmark feature of both HIV disease and NHL. However, the serum concentrations of circulating IFN- γ were positively associated with CD8 T-cells and NK-cells, thus it may enhance cytotoxic T-cell and NK-cell activities against HIV-1 infected cells and malignant lymphoma cells [Roff et al., 2014]. In addition, NK-cells are known to produce IFN- γ , thus the increased serum concentrations of circulating IFN- γ may have been caused by increased NK-cell expression.

7.5.2 Interleukin-1 beta (IL-1 β)

IL-1 β enhances antibody production of B-cells and promotes B-cell proliferation [Krause et al., 2012]. Elevated IL-1 β expression has been associated with several diseases including cancer [Krause et al., 2012; Karimbux et al., 2012]. In the current study, the serum concentrations of circulating IL-1 β in HIV+NHL were investigated. The serum concentrations of circulating IL-1 β were significantly reduced in HIV+NHL as compared to NHL and there was no significant difference when compared to HIV+ cART patients (figure 7.6). NHL patients had increased serum concentrations of circulating IL-1 β when compared to the controls (figure 7.6). IL-1 β is known to promote B-cell proliferation and differentiation hence its expression is increased in B-cell NHL [Krause et al., 2012]. HIV-1 infection did not influence the serum concentrations of circulating IL-1 β in NHL patients in the current study.

CART-naïve HIV+ patients had increased serum concentrations of circulating IL-1 β when compared to the controls (figure 7.6). In addition, cART-naïve HIV+ patients had elevated serum concentrations of circulating IL-1 β than HIV+ cART patients (figure 7.6). The observed increased serum concentrations of circulating IL-1 β may have been influenced by HIV-1 infection. It has been previously shown that the HIV-1 induces IL-1 β expression and this is associated with the progression of HIV disease [Guo et al., 2014]. It has been reported that HIV-1 interacts with chromosome 2 to inhibit IL-1 receptor antagonist, leading to increased IL-1 β production [Corley 2000]. cART has reduced the serum concentrations of circulating IL-1 β in HIV+ patients in the current study. High concentrations of IL-1 β have been observed in HIV-1 infection and cART reduces IL-1 β as a result of increased availability of IL-1 β

receptor antagonist [Connolly et al., 2005]. Sadeghi and colleagues [1995], demonstrated that cART suppresses the constitutive production of IL-1 β in HIV-1 infected patients. Increased serum concentrations of circulating IL-1 β were positively associated with CD8+CD38 expression in the present study (figure 7.7A). Thus it increased with increasing T-cell activation in HIV+ patients. Using HIV-1 transfected cultured human astrocytes, Mamik and Colleagues [2011] reported an increase in CD8+CD38 expression in IL-1 β activated astrocytes. Therefore, the increased serum concentrations of circulating IL-1 β observed in the current study, may have also led to increased T-cell activation. This may be detrimental to the immune system and may pinpoint the origin of chronic immune activation.

7.5.3 Interleukin-2 (IL-2)

IL-2 plays a role in the stimulation of activated T-cell proliferation, cytotoxic activity of CD8 T-cells and NK-cells [De Paoli 2001; Kedzierska and Crowe 2001]. In addition, IL-2 induces B-cells, and monocytes [De Paoli 2001; Kedzierska and Crowe 2001]. In the current study, the serum concentrations of circulating IL-2 in HIV+NHL were investigated. The serum concentrations of circulating IL-2 were significantly increased in HIV+NHL as compared to NHL (figure 7.8). As mentioned previously, IL-2 induces sCD23 expression, thus, the increased serum concentrations of circulating sCD23 that were observed in HIV+NHL patients may have been induced by increased IL-2 concentrations, thus contributing to chronic B-cell activation. However, there was no significant difference in serum concentrations of circulating

IL-2 between HIV+NHL and HIV+ cART patients. In addition, NHL had increased serum concentrations of circulating IL-2 as compared to the controls (figure 7.8), while cART-naïve HIV+ patients had higher serum concentrations of circulating IL-2 as compared to both HIV+ cART and the controls. These findings indicate that the serum concentrations of circulating IL-2 are greatly increased in NHL as well as in HIV+ state. In consistence, David and colleagues [1998], showed that IL-2 receptor expression in HIV+ patients with high viral load is greatly increased as compared to uninfected control individuals. In the same study, cART significantly reduced IL-2R expression in treated HIV+ patients [David et al., 1998]. IL-2 is involved in B-cell differentiation and proliferation, thus it may lead to expansion of EBV positive B-cells resulting in B-cell NHL development [de Lastours et al., 2014]. In a study conducted by Cozen and colleagues [2008], it was reported that IL-2 concentrations were significantly higher in lymphoma cases as compared to un-infected controls.

The serum concentrations of circulating IL-2 were negatively associated with the number of CD4 T-cells, NKT-cells and FoxP3 expression in the current study (figure 7.9A, C, and D), while they were positively correlated with increased numbers of CD8 T-cells and T-cell activation (figure 7.9B and E), confirming the increased immune activation that is stimulated by IL-2. It has been reported that IL-2 supports the growth of cytotoxic (CD8) T-cells and is essential for the induction of lymphokine activated killer cells [De Paoli 2001]. Thus the increased serum concentrations of circulating IL-2 may play a role in the anti-tumor activities. In addition, IL-2 stimulates the proliferation of activated T-cells, the observed increased serum concentrations may contribute to the chronic immune activation seen in both HIV-1 infection and

NHL. The increased IL-2 stimulates immune activation with CD8+CD38 and this is further promoted by reduced FoxP3 expression that normally regulates T-cell activation. cART may reduce the serum concentrations of circulating IL-2 in HIV+ patients which in turn decreases immune activation. In contrast to the current findings IL-2 therapy has been reported to increase CD4 T-cell counts in HIV-1 infected patients [Vento et al., 2006; Molina et al., 2009]. In addition, it has been reported that IL-2 therapy in HIV+ lymphoma patients may have a role in the prevention and treatment of HIV-1 associated lymphomas [Shah et al., 2000]

7.5.4 Interleukin-4 (IL-4)

IL-4 is known to stimulate B-cell activation and differentiation [Zamorano et al., 2003]. Its expression may explain the increased B-cell activation seen in HIV+NHL patients. In the current study, the serum concentrations of circulating IL-4 were significantly increased in HIV+NHL as compared to NHL as well as when compared to HIV+ cART patients (figure 7.10). In addition, the serum concentrations of circulating IL-4 were significantly increased in NHL as compared to the controls. These results suggest that the serum concentrations of circulating IL-4 are greatly increased in HIV+NHL as well as in the HIV negative NHL patients. Thus serum concentrations of circulating IL-4 are increased in NHL regardless of the HIV status and this is because IL-4 is needed for normal growth of B-cells [Lundin et al., 2001]. Gergely and colleagues [2004], reported that the frequency of CD4+ IL-4 expression is significantly higher in NHL patients as compared to controls and treatment with

CHOP reduces its expression. To determine the effect of HIV-1 infection as well as the effect of cART on the serum concentrations of circulating IL-4, cART-naïve HIV+ and HIV+ cART patients were compared. The serum concentrations of circulating IL-4 were significantly higher in cART-naïve HIV+ patients as compared to HIV+ cART patients, as well as when compared to the controls (figure 7.10). These results suggest that HIV-1 infection increases serum concentrations of circulating IL-4 and cART use decreases them. Using peripheral blood mononuclear cells, Valentin and colleagues [1998], showed that IL-4 activates HIV-1 expression and controls viral evolution and phenotypic switch that leads to accelerated disease progression. Kazazi and colleagues [1992], showed that IL-4 stimulates HIV-1 replication in the early phases of infection and may also facilitate virus transmission by aggregate formation.

The increased serum concentrations of circulating IL-4 may have led to chronic B-cell activation observed in NHL and HIV+ patients. In addition, increased serum concentrations of circulating IL-4 were associated with decreased numbers of CD4 T-cells, NKT-cells and FoxP3 expression (figure 7.11A, B and F), while they were also associated with increased numbers of NK-cells and CD8 T-cell activation (figure 7.11C and E). In addition, the serum concentrations of IL-4 were independently associated with HIV+NHL. This confirms the role played by HIV-1 in promoting immune activation while reducing immune tolerance and regulation.

7.5.5 Interleukin-6 (IL-6)

IL-6 has a wide variety of activities, including B-cell stimulation, monocyte differentiation and induction of IL-4 producing cells [Kedzierska and Crowe 2001; Connolly et al., 2005]. In addition, IL-6 is involved in tumor growth, malignant differentiation of cancer cells and immune-modulation of the micro-environment [Zarogoulidis et al., 2013]. In the current study, the serum concentrations of circulating IL-6 were significantly increased in HIV+NHL as compared to HIV+ cART patients, however, there was no significant difference when compared to NHL (figure 7.12). Although there was no significant difference in the serum concentrations of circulating IL-6 between HIV+NHL and NHL, there was a trend towards increased concentrations in HIV+NHL.

In addition, the serum concentrations of circulating IL-6 were significantly elevated in NHL when compared to the controls (figure 7.12). The increased serum IL-6 concentrations have been associated with elevated cancer risk, and these concentrations were found to be a prognostic factor for several cancer types [Zarogoulidis et al., 2013]. In addition, the elevated serum IL-6 concentrations have been previously found to be associated with the subsequent development of B-cell lymphomas in HIV-1 infected patients [Pluda et al., 1993]. Furthermore, Denizot and Colleagues [1996], showed that serum IL-6 expression is significantly higher in NHL patients as compared to the healthy controls. The effect of HIV-1 infection and cART on the serum concentrations of circulating IL-6 were also investigated in the current study.

The serum concentrations of circulating IL-6 were significantly increased in cART-naïve HIV+ patients as compared to HIV+ cART patients as well as when compared to the control individuals (figure 7.12). These results imply that HIV-1 infection increased the serum concentrations of circulating IL-6 and that cART use reduced them. This confirms that cART reduced HIV-1 viral load which decreased with serum concentrations of circulating IL-6. It has been shown that IL-6 can induce HIV-1 expression by acting at the transcriptional or post-transcriptional concentrations in infected monocytic cells [Poli et al., 1990]. During the acute stage of an infection, relatively high concentrations of IL-6 are produced and this is important in the activation of T-cells and increasing the number of antibody producing plasma cells against HIV-1 [Hosein 2012]. However, increased concentrations of IL-6 which induce B-cell activation may contribute to chronic B-cell activation.

The increased serum concentrations of circulating IL-6 were negatively associated with CD4 T-cell count, CD19, NKT and FoxP3 expression (figure 7.13A, B, C and E), while they were positively correlated with CD8+CD38 expression (figure 7.13D). These findings further confirm the increased B-cell activation in HIV+ patients. The negative associations with the numbers of CD4 T-cells, CD19+ B-cells, NKT-cells and FoxP3 expression observed in this study, suggest that IL-6 weakens the immune system of HIV+ patients. The increased T-cell activation with increasing IL-6 concentrations observed may also contribute to the depletion of the immune function, as it leads to immune exhaustion and T-cell turnover. It has been reported that chronically high concentrations of IL-6 may weaken the immune system in HIV-1 infected patients and these concentrations were also associated with 40% increased

risk for developing cancer [Hosein 2012]. Furthermore, high IL-6 expression has been associated with increased HIV-1 replication [Ullum et al., 1996], and the development of lymphoma in HIV-1 infected subjects [Poli et al., 1995].

7.5.6 Interleukin-8 (IL-8)

IL-8 is produced by tumor cells, and has been implicated to play a role in cancer progression [Xie 2001; Campbell et al., 2013]. In the current study, no significant difference in the serum concentrations of circulating IL-8 between HIV+NHL and NHL was observed (figure 7.14). However, as compared to HIV+ cART patients, HIV+NHL had significantly high serum concentrations of circulating IL-8 (figure 7.14). In addition, NHL had high serum concentrations of circulating IL-8 as compared to the controls (figure 7.14). These findings suggest that the serum concentrations of circulating IL-8 are increased in NHL regardless of the HIV status. It has been reported that the expression of IL-8 correlates with the angiogenesis, tumorigenicity, and metastatic potential of many solid cancers [Waugh and Wilson 2008].

It has also been suggested that targeting IL-8 signalling within the cancer cell micro-environment may assist in sensitizing cancer cells to conventional chemotherapy and novel treatment strategies [Waugh and Wilson 2008]. In addition, Sharma and Zhang [2001], showed that IL-8 is expressed in HIV associated lymphoma B-cell lines. Engel and Colleagues [2012], reported that viral IL-8 promotes lymphomagenesis through targeted recruitment of B-cells. Consistent with the current findings, Denizot and Colleagues [1996] showed that serum IL-8

concentrations are significantly higher in NHL patients as compared with the healthy controls. In the current study, the serum concentrations of circulating IL-8 were significantly increased in cART-naïve HIV+ patients as compared to HIV+ cART as well as when compared to the controls (figure 7.14). This indicates that the serum concentrations of circulating IL-8 are increased in HIV+ state, and cART may reduce them.

The serum concentrations of circulating IL-8 were negatively associated with the numbers of CD4 T-cells, CD19+ B-cells and NKT-cells in HIV+ patients (figure 7.15A, B, and C). In addition, the serum concentrations of circulating IL-8 were positively associated with CD8 T-cell activation (figure 7.15D). It has been reported that the serum concentrations of IL-8 are elevated in the peripheral circulation of HIV-1 infected patients [Taylor 1998]. In a study by Lane and Colleagues [2001], it has been shown that IL-8 expression is increased in lymphatic micro-environment in HIV-1 infected patients and that inhibition of the activity of endogenous IL-8 markedly reduces HIV-1 replication. This suggests that IL-8 may play a role in HIV-1 replication and disease progression.

7.5.7 Interleukin-10 (IL-10)

IL-10 is known to have multiple effects on B-cells, including stimulation of growth and differentiation [Blay et al., 1993; Masood et al., 1995]. In the current study, the serum concentrations of circulating IL-10 were significantly higher in HIV+NHL as compared to HIV+ cART patients, however, there was no significant difference when HIV+NHL patients were compared to NHL patients (figure 7.16). In addition, NHL patients had increased serum concentrations of circulating IL-10 as compared to the controls (figure 7.16). These findings suggest that the serum concentrations of circulating IL-10 are increased in HIV+NHL patients. Voorzanger and Blay [1996], previously reported that HIV+NHL may produce higher amounts of IL-10 than HIV negative NHL patients and HIV may be directly responsible for an increased IL-10 secretion.

Consistent with the current findings, Gupta and colleagues [2012], demonstrated that serum IL-10 concentrations are significantly higher in a subset of DLBCL patients as compared to controls and were correlated with adverse clinical features and shorter event free survival. The observed increased serum concentrations of circulating IL-10 may have contributed to the development of NHL. In a Multicenter AIDS Cohort Study, Breen and Colleagues [2003], showed that elevated serum IL-10 concentrations are associated with the development of lymphoma in HIV-1 infected individuals. In the same study, detectable serum IL-10 was seen much more frequently in lymphoma cases as compared to both HIV-1 infected patients and healthy controls [Breen et al., 2003]. Furthermore, in a recent study conducted by Edlefsen and colleagues [2014], the increased risk of DLBCL development was observed in women with increased IL-10 expression.

In addition, it has been previously shown that IL-10 production contributes to the clinical course of DLBCL and this phenomenon involves a substantial genetic component [Masood et al., 1995; Lech-Maranda et al., 2004; Bogunia-Kubik et al., 2008; Hosgood et al., 2013]. cART-naïve HIV+ patients had significantly higher serum concentrations of circulating IL-10 than both HIV+ cART patients and controls (figure 7.16) in the current study. Thus, HIV-1 infection may have been a driving force to increased serum concentrations of circulating IL-10.

The serum concentrations of circulating IL-10 are greatly increased in cART-naïve HIV+ patients by nearly 4 fold. cART initiation decreased the serum concentrations of circulating IL-10 in HIV+ patients. The increased serum concentrations of circulating IL-10 were positively associated with the numbers of NK-cells and T-cell activation (figure 7.17A and B). In consistence with the current findings, Brockman and Colleagues [2009] showed that IL-10 mRNA expression and plasma IL-10 concentrations were increased in the setting of chronic uncontrolled HIV-1 infection and were correlated with plasma viremia. In the same study, both IL-10 mRNA expression and plasma concentrations were reduced through successful cART treatment [Brockman et al., 2009].

7.5.8 Interleukin-12p70 (IL-12p70)

IL-12p70 plays an important role in anti-tumor activities [Toubai et al., 2006; Pan et al., 2012]. The serum concentrations of circulating IL-12p70 were investigated in the current study. No statistical significant difference in the serum concentrations of circulating IL-12p70 between HIV+NHL and NHL patients was found (figure 7.18). However, there was a trend towards increased serum concentrations of circulating IL-12p70 in NHL. In addition, HIV+NHL had significantly lower serum concentrations of circulating IL-12p70 as compared to HIV+ cART patients (figure 7.18). These findings suggest that the serum concentrations of circulating IL-12p70 are decreased in HIV+NHL, and this may result in decreased anti-tumor activity against malignant lymphoma cells leading to its pathogenesis and progression.

As compared to the controls, NHL patients had increased serum concentrations of circulating IL-12p70 (figure 7.17). In HIV negative NHL, anti-tumor activities are required to destroy malignant lymphoma cells, thus the increased serum concentrations of circulating IL-12p70 indicate increased anti-tumor activities which is beneficial for these patients. There was no significant difference in the serum concentrations of circulating IL-12p70 between HIV+ cART and cART-naïve HIV+ (figure 7.18). cART-naïve HIV+ patients had increased serum concentrations of circulating IL-12p70 as compared to the controls (figure 7.18). These results suggest that the serum concentrations of circulating IL-12p70 are increased in untreated HIV+ patients. Consistent with the current findings, Roskstroh and Colleagues [1998], showed that serum IL-12p70 concentrations are significantly increased in HIV-1 infected patients as compared to healthy controls. IL-12p70 may be playing a

role in the eradication of HIV-1 virus. In a study by Roberts and Colleagues [2010], it was shown that higher concentrations of IL-12p70 observed in HIV-1 infected patients are associated with lower viral load. The serum concentrations of circulating IL-12p70 were negatively associated with the numbers of CD4 T-cells, CD19+ B-cells, and FoxP3 expression (figure 7.19A, C, and F), while they were positively associated with the numbers of CD8 T-cells, NK-cells, and CD8+CD38 expression (figure 7.19B, D, and E) in the current study.

IL-12p70 is known to play a critical role in the generation of cell mediated immune responses to infectious agents including HIV-1 [Guzzo et al., 2011], thus the association of IL-12p70 with the numbers of CD8 T-cells and NK-cells observed in the current study confirm this role. IL-12p70 may stimulate the activities of CD8 and NK-cells as confirmed by positive correlation of IL-12p70 with increased CD8+CD38 expression. This may also explain the increased serum concentrations of circulating IL-12p70 in NHL patients observed in the present study which may increase cell mediated immunity against lymphoma cells.

7.5.9 Interleukin-13 (IL-13)

IL-13 enhances antigen presentation in HIV-1 infected patients, and induces the differentiation and function of macrophages [Bailer et al., 1999; Jensen 2000]. IL-13 stimulates B-cell growth and immunoglobulin class switching of B-cells [Skinnider et al., 2001]. In the current study, the serum concentrations of circulating IL-13 were investigated. No statistical significant difference was observed in the serum concentrations of circulating IL-13 between HIV+NHL and NHL as well as when HIV+NHL were compared to HIV+ cART patients (figure 7.20). However, NHL patients had increased serum concentrations of circulating IL-13 as compared to the controls (figure 7.20). It has been reported that IL-13 expression is increased in NHL patients and may provide growth and survival advantage to B-cell NHL [Billard et al., 1997]. These findings suggest that the increased serum concentrations of circulating IL-13 may have increased the growth of B-cells in NHL patients. HIV-1 infection did not influence the serum concentrations of circulating IL-13 in NHL groups.

Furthermore, no significant difference was found in the serum concentrations of circulating IL-13 between HIV+ cART and cART-naïve HIV+ patients (figure 7.20). However, cART-naïve HIV+ patients had increased serum concentrations of circulating IL-13 as compared to the controls (figure 7.20). These results indicate that the serum concentrations of circulating IL-13 are increased in untreated HIV+ patients. The serum concentrations of circulating IL-13 were negatively associated with the numbers of CD4 T-cells (figure 7.21A), while they correlated positively with CD8+CD38 expression (figure 7.21B). It has been shown that IL-13 activates CD8 T-cells and NK-cells and increases HIV-1 specific cell mediated responses [Emille et

al., 1997; Papasavvas et al., 2005], therefore, the observed increased serum concentrations of circulating IL-13 in cART-naïve HIV+ patients may have been triggered by HIV-1 virus. By increasing HIV-1 specific cell mediated immune responses, IL-13 may reduce the viral load. Montaner and Colleagues [1993] reported that IL-13 suppresses HIV-1 infection within monocytes and macrophages in vivo. The increased serum concentrations of circulating IL-13 were positively correlated with increased CD8+CD38 in the current study, confirming that IL-13 enhances CD8 T-cell activation. Thus, the serum concentrations of circulating IL-13 may have increased with increasing T-cell activation which resulted in the reduction in CD4 T-cell counts.

7.5.10 Tumor necrosis factor- α (TNF- α)

TNF- α is involved in pathological processes such as chronic inflammation, autoimmunity and malignant diseases [Balkwill 2006]. In the current study, the serum concentrations of circulating TNF- α were investigated in HIV+NHL patients. The serum concentrations of circulating TNF- α were significantly increased in HIV+NHL patients as compared to NHL patients (figure 7.22). However, no significant difference in TNF- α was found between HIV+NHL and HIV+cART patients. In addition, NHL patients had increased serum concentrations of circulating TNF- α as compared to the controls (figure 7.22). It has been previously shown that genetic polymorphism leading to increased TNF- α production influences the clinical outcome of NHL and suggest a pathophysiological role for the genetic control of the immune

response in lymphomas [Warzocha et al., 1998; Ibrahim et al., 2012]. In addition, it has been shown that patients with malignant lymphomas have high circulating concentrations of TNF- α and that higher plasma concentrations of TNF- α are associated with poor disease outcome [Salles et al., 1996; Warzocha et al., 1998]. cART-naïve HIV+ patients had significantly high serum concentrations of circulating TNF- α as compared to the HIV+ cART patients as well as when compared to the controls (figure 7.22). These results confirm that TNF- α is increased in HIV+ patients, while cART decreased the serum concentrations of circulating TNF- α to almost the level of the control population. The serum concentrations of circulating TNF- α were increased in NHL without HIV-1 infection.

The increased serum concentrations of circulating TNF- α were negatively associated with the numbers of CD4 T-cells, CD19+ B-cells, and FoxP3 expression (figure 7.23A, D, and F), while they correlated positively with the numbers of CD8 T-cells, NK-cells and CD8+CD38 expression (figure 7.23B, C and E). In addition, TNF- α was independently associated with HIV+NHL. It has been previously shown that HIV-1 infection induces TNF- α expression, and the increased TNF- α expression is associated with increased viral replication, depletion of CD4 T-cell counts and poor outcome in HIV-1 infected patients [Kumar et al., 2013]. It has also been reported that the expression of TNF- α is greatly increased in HIV-1 infection and that these levels increase with disease progression [Kedzierska and Crowe 2001; Connolly et al., 2005; Ownby et al., 2009]. HIV-1 induces TNF- α expression, and exogenous TNF- α enhances HIV-1 replication and positive correlation between increased serum

concentrations of TNF- α and increased plasma HIV-1 viral load have been demonstrated [Connolly et al., 2005].

7.6 Conclusion

HIV-1 infection is associated with dysregulation of cytokine production and this is thought to contribute to HIV associated immune deficiency [Kedzierska and Crowe 2001; Shebl et al., 2012]. It has been reported that decreased secretion of specific cytokines and increased production of others contributes to the progression of HIV-1 associated immune deficiency [Kedzierska and Crowe 2001]. The dysregulation of cytokine production may be playing a role in the pathogenesis of HIV+NHL, as evidenced by increased prevalence of NHL in HIV-1 infection.

In the current study, the concentrations of inflammatory cytokines were increased in HIV+NHL. This may have been a carry-over effect from increased concentrations observed in HIV+ patients and may have been caused by HIV-1 infection. However, high serum concentrations of circulating inflammatory cytokine were also observed in HIV negative NHL patients. This may have been caused by the presence of EBV virus in these NHL patients or immune surveillance against malignant lymphoma cells. cART partially decreased the serum concentrations of circulating inflammatory cytokines. Thus in the current study, cART reversed the abnormal cytokine profile and this may contribute to suppression of HIV-1 replication and restoration of CD4 T-cell counts.

The decreased concentrations of inflammatory cytokines and B-cell activation that was observed following cART initiation, may have resulted primarily from the virological suppression of HIV-1 virus mediated by cART. It is evident that local inflammatory processes and antigenic drive by HIV-1 can promote lymphomagenesis at the site of inflammation and chronic immune activation [Baecklund et al., 2014]. Cytokines play an important role in the pathogenesis of lymphomas and may contribute to the clinical manifestations in HIV+NHL [Talaat et al., 2014].

CHAPTER EIGHT

SUMMARY AND OVERALL CONCLUSION

8.1 Summary Results

In the current study, HIV+NHL patients had suppressed CD4 T-cells, CD19+ B-cells, NKT-cells and FoxP3 expression (Table 8.1). However, CD8, NK-cells, CD8+CD38 (Table 8.1), markers of chronic B-cell activation (Table 8.2) and inflammatory cytokines (IFN- γ , IL-2, IL-4, IL-6, IL-13 and TNF- α) (Table 8.3) were greatly increased in HIV+NHL patients as compared to all other groups except cART-naïve HIV+ patients. Reduced numbers of CD4 T-cells, NKT-cells and FoxP3 expression were associated with increased B-, T-cell activation and inflammatory markers. No significant association was observed between the biomarkers of B-cell activation and inflammatory markers.

Table 8.1: Summary results of basic and T-cell activation biomarkers. Mean \pm SD.

<i>Biomarker</i>	HIV+NHL	NHL	HIV+ cART	cART-naïve HIV+	CONTROLS
CD4 (Cells/mm ³)	298 \pm 218	537 \pm 375	417 \pm 260	238 \pm 153	1101 \pm 284
CD8 (Cells/mm ³)	701 \pm 377	589 \pm 503	544 \pm 271	811 \pm 403	426 \pm 124
CD19 (Cells/mm ³)	238 \pm 200	277 \pm 262	262 \pm 189	108 \pm 73	358 \pm 138
NK (Cells/mm ³)	87 \pm 47	62 \pm 55	51 \pm 39	817 \pm 37	37 \pm 30
NKT (Cells/mm ³)	161 \pm 102	230 \pm 177	238 \pm 213	217 \pm 197	334 \pm 153
CD8+CD38 (%)	10.8 \pm 7.80	9.56 \pm 5.53	7.36 \pm 6.90	15.95 \pm 8.81	3.65 \pm 1.48
FoxP3 (%)	4.28 \pm 1.87	6.37 \pm 2.04	5.02 \pm 0.91	4.02 \pm 1.28	7.59 \pm 1.70

Table 8.2: Summary results of B-cell activation markers. Mean \pm SD.

<i>Biomarker</i>	HIV+NHL	NHL	HIV+ cART	cART-naïve HIV+	CONTROLS
sCD20 (ng/ml)	5.62 \pm 1.69	3.92 \pm 0.63	4.75 \pm 1.34	5.11 \pm 1.49	3.04 \pm 0.84
sCD23 (U/ml)	204.63 \pm 92.12	148.76 \pm 94.28	139.20 \pm 130.83	69.28 \pm 48.90	94.32 \pm 35.54
sCD27 (ng/ml)	22.80 \pm 11.20	22.28 \pm 12.87	13.71 \pm 4.09	19.74 \pm 9.48	12.21 \pm 1.87
sCD30 (pg/ml)	577.41 \pm 256.31	384.50 \pm 176.07	274.11 \pm 266.13	265.32 \pm 69.91	239.98 \pm 117.04
sCD44 (ng/ml)	7.25 \pm 1.23	6.03 \pm 1.41	4.84 \pm 1.57	6.08 \pm 2.61	4.30 \pm 1.37

Table 8.3: Summary results of inflammatory markers. Mean \pm SD.

<i>Biomarker</i>	HIV+NHL	NHL	HIV+ cART	cART-naïve HIV+	CONTROLS
IFN- γ (pg/ml)	26.719 \pm 25.632	20.425 \pm 29.907	10.458 \pm 8.920	33.067 \pm 9.340	5.734 \pm 2.328
IL-1 β (pg/ml)	0.117 \pm 0.083	0.196 \pm 0.096	0.113 \pm 0.079	0.201 \pm 0.180	0.102 \pm 0.078
IL-2 (pg/ml)	0.356 \pm 0.135	0.249 \pm 0.116	0.328 \pm 0.148	0.714 \pm 0.338	0.077 \pm 0.044
IL-4 (pg/ml)	0.102 \pm 0.036	0.081 \pm 0.016	0.081 \pm 0.026	0.112 \pm 0.042	0.056 \pm 0.030
IL-6 (pg/ml)	1.473 \pm 1.256	1.179 \pm 1.171	0.779 \pm 0.268	2.447 \pm 1.350	0.415 \pm 0.190
IL-8 (pg/ml)	13.027 \pm 5.341	13.942 \pm 7.602	9.615 \pm 5.483	14.002 \pm 3.649	9.110 \pm 2.733
IL-10 (pg/ml)	1.119 \pm 0.691	1.344 \pm 1.550	0.482 \pm 0.210	1.312 \pm 0.569	0.375 \pm 0.338
IL-12p70 (pg/ml)	0.198 \pm 0.115	0.250 \pm 0.143	0.459 \pm 0.226	0.576 \pm 0.245	0.101 \pm 0.091
IL-13 (pg/ml)	0.920 \pm 0.437	0.776 \pm 0.249	0.870 \pm 0.439	0.955 \pm 0.285	0.563 \pm 0.358
TNF- α (pg/ml)	2.012 \pm 1.581	1.144 \pm 0.394	1.342 \pm 0.465	3.198 \pm 2.029	0.925 \pm 0.268

8.2 Overall conclusion

HIV+NHL patients had suppressed numbers of CD4 T-cells and NKT cells, and increased numbers of NK-cells and CD8 T-cells in the current study. However no significant difference in the numbers of CD19+ B-cells was observed. The reduced numbers of CD4 T-cells and NKT-cells may have been caused by HIV-1 infection. The main target of HIV-1 infection is CD4 T-cells and it leads to the reduction in CD4 T-cell counts [Hogg et al., 2001; Alimonti et al., 2003; Fasakin et al., 2014]. Chronic immune activation observed in HIV-1 infected patients may also lead to increased CD4 T-cell turnover and apoptosis [Haas et al., 2011; Massanella et al., 2013]. The reduction in CD4 T-cell counts can expose HIV+ patients to opportunistic infections including EBV. Low CD4 T-cell counts and detectable serum EBV load are independently associated with poor survival among HIV+NHL patients [Tedeschi et al., 2012]. However, chemotherapy may have also led to decreased CD4 T-cell counts especially in the first few months of treatment [Mackall et al., 1994; Proietti et al., 2012; Nars and Kaneno 2013].

In addition, while the numbers of CD4 T-cells decreases, the numbers of CD8 T-cells and NK-cells increases in HIV+NHL patients. This may be due to the immune system trying to eradicate the HIV-1 virus, EBV and to destroy malignant lymphoma cells. In the current study, cART increased the numbers of CD4 T-cells while reducing the numbers of CD8 T-cells and NK-cells and this may be due to the depletion of viral load [Smith et al., 2003; Wolbers et al., 2007]. The numbers of CD8 T-cells and NK-cells is mediated by HIV-1 RNA levels [Catalfamo et al., 2008]. The continued presence of the HIV-1 virus causes the sustained immune activation, as the immune

system is trying to fight the infection [Cao et al., 2009; Rajasuriar et al., 2013]. Unfortunately, chronic immune activation results in HIV-1 complications [Cao et al., 2009; Catalfamo et al., 2011]. This includes increased T-cell turn-over rate and immune exhaustion which is the main cause of immune deficiency in this group of patients [Haas et al., 2011; Catalfamo et al., 2011; Massanella et al., 2013]. T-cell activation was greatly increased in HIV+NHL and cART-naïve HIV+ patients in the current study, as evidenced by increased CD8+CD38 expression. The increased T-cell activation observed in the current study may have been caused by HIV-1 infection, the presence of EBV, and anti-tumor immune response against malignant lymphoma cells. However, T-cell activation was negatively associated with CD4 T-cell counts, thus it may have resulted in increased CD4 T-cell turnover and immune exhaustion.

Also observed in the current study was decreased regulatory T-cells as evidenced by reduced FoxP3 expression in HIV+NHL. Depleted FoxP3 expression was associated with increased T-cell activation. The current findings indicate that chronic immune activation may have been a result of decreased immune regulation in HIV+NHL patients. Immune regulation is necessary in the control of immune activation and the prevention of auto immunity. T-reg cells are known to suppress T-cell activation, proliferation and cytokine production [Card et al., 2009; Presicce et al., 2011]. In the absence of immune regulation, sustained immune activation occurs without monitoring. In addition, cART use was associated with improved immunity, increased regulatory T-cells, and decreased T-cell activation.

Biomarkers of B-cell activation (sCD20, sCD23, sCD27, sCD30, and sCD44) were elevated in HIV+NHL and cART-naïve HIV+ patients in the present study. This is inconsistent with previous findings. Breen and Colleagues [2011], reported that serum sCD23, sCD27 and sCD30 concentrations were significantly elevated in HIV+NHL as compared to HIV+ controls. In the current study, it was shown that chronic B-cell activation also occurred in untreated HIV+ patients and cART may reduce B-cell activation. Increased B-cell activation has been observed in HIV-1 infected patients and was associated with a more rapid disease progression and poor survival [Horie and Watanabe 1998]. These results also suggest that B-cell activation is increased in HIV+NHL as evidenced by increased B-cell activation markers investigated in this study, and was associated with decreased CD4 T-cell counts and increased T-cell activation.

The increased T-cell activation observed in HIV+NHL may have also caused chronic B-cell activation. It has been reported that chronic B-cell activation may be caused by related interaction with activated T-cells, whose receptor recognizes antigen presented by the B-cells, or activation by T-cell independent antigens [Bishop et al., 2003]. In addition, there is growing evidence that HIV-1 virus can directly contribute to B-cell activation via direct interactions with B-cells [Epeidegui et al., 2010]. Chronic B-cell activation is known to increase the risk of HIV+NHL development [Grulich et al., 2000; Purdue et al., 2009; De Roos et al., 2012]. It has been previously shown that elevated serum concentrations of sCD23, sCD27 and sCD30 are associated with subsequent diagnosis of HIV+NHL [De Roos et al., 2012; Hussain et al., 2013]. The downstream effects of chronic B-cell activation with

ongoing engagement of the B-cell receptor complex on lymphomagenesis are numerous, and include the accumulation of oncogene mutations and translocations resulting from aberrant expression and gene targeting of the DNA mutating enzyme, activation induced cytidine deaminase (AICDA) [Hussain et al., 2013]. B-cell activation leads to the expression of AICDA, a DNA editing enzyme that mediates immunoglobulin gene class switch recombination and somatic hypermutation [Vendrame et al., 2014]. It has been shown that AICDA is over expressed before the development of HIV+NHL which is consistent with a direct role for this molecule in the pathogenesis of NHL [Vendrame et al., 2014].

HIV-1 infection has long been associated with dysregulation of cytokine production and this may contribute to the immune deficiency observed in HIV-1 infected patients [Kedzierska and Crowe 2001; Shebl et al., 2012]. The serum concentrations of circulating inflammatory cytokines were greatly increased in HIV+NHL patients in the current study. The increased serum concentrations of circulating inflammatory cytokines observed in the current study may contribute to establishing chronic immune activation that exacerbates HIV+NHL [Roff et al., 2014]. Vendrame and Colleagues [2014] showed that elevated serum concentrations of cytokines and biomarkers of inflammation and immune activation precedes the development of HIV+NHL. The increased serum concentrations of circulating inflammatory cytokines were positively associated with the numbers of CD8 T-cells, NK-cells and T-cell activation. Persistent HIV-1 infection causes chronic immune activation (B and T-cell activation) which in turn results in increased pro-inflammatory cytokine production. The increased serum concentrations of circulating inflammatory cytokines may have

been caused by chronic T-cell activation as cytokines are secreted by activated T-cells. These inflammatory cytokines may have enhanced cytotoxic T-cell and NK-cell activities against HIV-1 infected cells and malignant lymphoma cells thus playing a role in antitumor activities [Roff et al., 2014]. Inflammatory cytokines such as IL-1 β , IL-4, IL-6, and IL-10 are known to stimulate B-cell activation, proliferation and differentiation, thus may contribute to B-cell activation [Zamorano et al., 2003; Krause et al., 2012; Connolly et al., 2005; Masood et al., 1995]. In addition, the increased cytokine concentrations, together with the B-cell activation driven by antigen exposure and the direct stimulation of B-cells by HIV-1, can result in chronic B-cell hyperactivation [Rabkin et al., 2011]. The cytokine mediated hyperstimulation of B-cell proliferation may play a role in HIV associated lymphomagenesis [Rabkin et al., 2011]. It is well established that cytokines play a role in tumor growth, malignant differentiation of cancer cells and immune-modulation of micro-environment [Zarogoulidis et al., 2013].

There are two major mechanisms that appear to be involved in lymphomagenesis in HIV-1 infected individuals: loss of immunoregulatory control of EBV and chronic B-cell activation due to the immune dysfunction resulting from HIV-1 infection [Regidor et al., 2011]. However, whether these biomarkers lead to the development of HIV+NHL was not investigated in the current cross-sectional study. Previous studies showed that higher levels of T-cell activation, T_H1 cytokine serum concentration and markers of EBV replication, preceded B-cell lymphoma diagnosis [Breen et al., 2003; Ouedraogo et al., 2013]. The HIV-1 infection which is well known for down-regulating CD4 T-cell counts causes immune deficiency and this appears to contribute to the

pathogenesis of HIV+NHL through the loss of T-cell mediated control over B-cell proliferation and the loss of immune-regulatory control over EBV infected B-cells [Hussain et al., 2013; Nolen et al., 2014]. It has been previously shown that dysregulated cytokine expression may be involved in the development of B-cell NHL [Gu et al., 2010; Saberi Hoenijeh et al., 2010]. Furthermore, the serum concentrations of circulating IL-12p70 that plays an important role in anti-tumor activities [Toubai et al., 2006; Pan et al., 2012], were decreased in HIV+NHL patients and this may result in decreased anti-tumor activities against HIV+NHL leading to its pathogenesis and progression.

cART treatment was associated with a decrease in serum concentrations of circulating inflammatory cytokines in the current study. These findings confirm that the biomarkers of immune activation and inflammation are increased in the South African population as was seen in other regions. FoxP3, sCD20, sCD23, sCD44, IL-4 and TNF- α were independently associated with HIV+NHL. The selected biomarkers of immune activation and inflammation investigated in the current study will enhance our ability to diagnose, monitor and treat HIV+NHL.

8.3 Limitations of the study

The current study had limitations. The sample size was small; however, this was substantiated by formal statistical sample size calculation which was confirmed by the fact that significant differences and associations have been detected in the study. No follow-up assessment of these biomarkers was conducted. It would have been worthwhile to conduct a follow-up assessment, to investigate whether the expression of these biomarkers are time dependent or not. This is a cross-sectional study and therefore no conclusions about causality can be drawn.

8.4 Future studies

A follow-up assessment on these biomarkers needs to be conducted in a longitudinal study, to investigate whether their expression is time dependent. Based on the current findings, the following biomarkers should be taken forward for longitudinal analysis, i.e FoxP3, sCD20, sCD23, sCD44, IL-4 and TNF- α . Biomarkers that carry the best prognostic value in predicting the possibilities of the patients to develop HIV+NHL need be determined.

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APPENDIX I

INCLUSION CRITERIA

- cART-naïve HIV+
 - HIV positive patients with CD4 T-cell count of ≤ 350 cells/mm³
 - Not yet on cART
 - No medication such as immune suppressants and those for autoimmune diseases
- HIV+ cART
 - HIV positive patients with CD4 T-cell count of ≤ 350 cells/mm³ that are on cART
 - First or second line combination antiretroviral therapy (ART) regimens according to SA clinical guidelines including efavirenz, nevirapine, tenofovir, lamuvidine, emtracitabine, stavudine, zidovudine and lopinavir/ritonavir.
 - Patients on other cART treatments may also be included in this study.
 - No medication for autoimmune diseases
- NHL
 - HIV negative NHL patients diagnosed with DLBCL that are on:
 - Defined chemotherapy such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) and supportive drugs
 - No other medication especially that may alter biomarker concentrations

- HIV+NHL
 - HIV positive NHL patients diagnosed with DLBCL that are on:
 - First or second line combination antiretroviral therapy (cART) regimens according to SA clinical guidelines including efavirenz, nevirapine, tenofovir, lamuvidine, emtracitabine, stavudine, zidovudine and lopinavir/ritonavir.
 - Defined chemotherapy such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) and supportive drugs
 - Patients on other cART treatments may also be included in this study.
 - No other medication especially that may alter biomarker concentrations
- Controls (Age/gender matched with the HIV+/NHL group)
 - HIV negative individuals with no NHL
 - No cART
 - No chemotherapy
 - No medication such as immune suppressants and those for autoimmune diseases
 - HIV test, counselling and referrals will be done on healthy control (Medical Students, laboratory and hospital staff) prior to the enrolment into the study.

Appendix II

Multitest antibody preparation:

- Multitest CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC
- Multitest CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC

Calculation of dilution factors: **1:3** (1 μ l reagent: in 3 μ l of ddH₂O)

Required volume: 20 μ l per sample and CD Chex (e.g. 4 samples and 2 CD Chex)

$$20\mu\text{l} \times 6 = 120\mu\text{l}$$

$$120\mu\text{l} + 20\mu\text{l (compensation volume)} = 140\mu\text{l}$$

$$1/3 \times 140\mu\text{l} = 47\mu\text{l}$$

47 μ l reagent + 93 μ l ddH₂O

Appendix III

FoxP3 Buffer A preparation:

- FoxP3 Buffer A (10x concentration)

Calculation of dilution factors: **1:10** (1µl reagent: 10µl of ddH₂O)

Required volume: 500µl per sample and lymphosures (e.g. 4 samples and 2 lymphosures)

$$500\mu\text{l} \times 6 = 3000\mu\text{l}$$

$$3000\mu\text{l} + 300\mu\text{l (compensation volume)} = 3300\mu\text{l}$$

$$1/10 \times 3300\mu\text{l} = 330\mu\text{l}$$

$$\text{Buffer A} = 330\mu\text{l Buffer A} + 2970\mu\text{l ddH}_2\text{O}$$

FoxP3 Buffer C preparation:

- FoxP3 Buffer B
- Prepared FoxP3 Buffer A (1x)

Calculation of dilution factors: **1:50** (1µl Buffer B: 50µl Buffer A)

Required volume: 500µl per sample and lymphosures (e.g. 4 samples and 2 lymphosures)

$$500\mu\text{l} \times 6 = 3000\mu\text{l}$$

$$3000\mu\text{l} + 300\mu\text{l (compensation volume)} = 3300\mu\text{l}$$

$$1/50 \times 3300\mu\text{l} = 66\mu\text{l}$$

$$3300\mu\text{l} - 66\mu\text{l} = 3234\mu\text{l}$$

$$\text{Buffer C} = 66\mu\text{l Buffer B} + 3234\mu\text{l Buffer A}$$