The interaction between DC-SIGN and DC-SIGNR with HHV-8 (LANA-1) and HIV-p24 in Castleman disease

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Signed by candidate
DEDICATION

I dedicate this research project to my beautiful son Kaiyur for making me believe that nothing is impossible; and to my dad Rama Chetty, thank you for everything.
ACKNOWLEDGEMENTS

I would also like to express my gratitude to the following people:

1. My supervisor, Prof Dhiren Govender for his continuous encouragement, patience and support.
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4. And finally my much adored husband, Nishen Brijraj, for understanding my academic aspirations and for being my rock as I juggled both career and family.
ABSTRACT

Background
Castleman disease (CD) is a lymphoproliferative disorder with four subtypes, some of which are aetiologically linked to Human Herpes virus 8 (HHV-8) which is known to cause diseases preferentially occurring in HIV-infected individuals. There has been a notable increase in the number of patients with HIV/HHV-8 associated CD diagnosed in the Groote Schuur hospital complex.

Aims
The aim of the study was to determine the role of DC-SIGN, DC-SIGNR, p24 and HHV-8 (LANA-1) in Castleman disease. Our objectives were to identify the presence of DC-SIGN and DC-SIGNR in HHV-8 infected cells, determine whether HHV-8 and p24 (HIV) co-infection occurs in the same cells and to determine whether HHV-8 infects B and/or T cells. This study not only represents the largest and first immunophenotypic investigative evaluation of CD but also signifies the first double staining immunohistochemical analysis of CD diagnosed at Groote Schuur hospital.

Methods
This was both a retrospective descriptive as well as an analytic cross-sectional immunohistochemistry study. Fifty cases of CD diagnosed at the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur hospital over a ten and half year period were included in the study. Double immunohistochemistry was used to characterise HHV-8 infected cells using LANA-1 antibody, in conjunction with DC-SIGN, DC-SIGNR, p24, CD20 and CD3. Immunophenotypic analysis was then performed to assess 1) the number of infected HHV-8 cells and 2) number and distribution of cells co-expressing HHV-8 and DC-SIGN, DC-SIGNR, p24, CD20 and CD3. The immunophenotypic profiles were then compared to the CD morphologic subtypes.

Results
The study cohort included 26 male and 24 female patients (M: F = 1.08:1), mean age 37.7 years. There were 16 hyaline vascular CD (HV-CD), 16 plasmablastic CD (Pb-CD). Nine plasma cell CD and 9 mixed-CD subtypes. There was a statistically significant association between HIV (n=45) and HHV-8 (n=40) positivity (p < 0.0002). CD4 counts and HAART enrolment were not predictive of CD development (p = 0.6120). Concurrent Kaposi sarcoma was seen in 16% (n=8) of the cohort.
When comparing Pb-CD and HV-CD, there were statistically significant differences in density of LANA-1 infected cells (p<0.0002), LANA-1/DC-SIGN co-expressing cells (p <0.0072) and LANA-1/p24 co-expressing cells (p<0.0001).

**Conclusions**

The findings of this study suggest that DC-SIGN may have a role in HHV-8 entry into cells. Furthermore, there is evidence that HIV and HHV-8 co-infection may function synergistically in CD. It is possible that DC-SIGN and DC-SIGNR facilitate dual viral entry into cells and influence viral replication and persistent infection.
TABLE OF CONTENTS

DECLARATION................................................................................................................. ii
DEDICATION.................................................................................................................. iii
ACKNOWLEDGEMENTS.................................................................................................. iv
ABSTRACT ...................................................................................................................... v
LIST OF FIGURES .......................................................................................................... x
LIST OF TABLES ............................................................................................................. xi
LIST OF ABBREVIATIONS ........................................................................................... xii
CHAPTER 1: .................................................................................................................... 1
LITERATURE REVIEW .................................................................................................... 1
1.1 Introduction ............................................................................................................. 1
1.2 Historical perspective ............................................................................................ 3
1.3 Classification .......................................................................................................... 4
1.3.1 Current classification ....................................................................................... 4
1.3.2 Proposed classification .................................................................................... 5
1.4 Pathology ................................................................................................................ 5
1.4.1 Hyaline vascular subtype ............................................................................... 5
1.4.2 Plasma cell subtype ......................................................................................... 6
1.4.3 Plasmablastic subtype .................................................................................... 7
1.4.4 Mixed subtype ................................................................................................ 8
1.4.5 Idiopathic multicentric Castleman Disease ..................................................... 8
1.5 Epidemiology of unicentric (UCD) and multicentric (MCD) .................................. 10
1.6 Pathogenesis of CD .............................................................................................. 12
1.6.1 Human Herpes virus 8 .................................................................................... 12
1.6.2 HIV .................................................................................................................. 18
1.6.3 Cytokine factors ............................................................................................. 19
1.6.4 Growth factors ............................................................................................... 21
1.6.5 B-cell populations .......................................................................................... 22
1.7 DC-SIGN .............................................................................................................. 24
1.7.1 Functions of DC-SIGN ................................................................................... 24
1.7.2 DC-SIGN and HIV ........................................................................................ 25
1.7.3 DC-SIGN and HHV-8 ..................................................................................... 26
1.7.4 DC-SIGNR ..................................................................................................... 28
1.8 HIV/HHV-8-associated MCD ............................................................... 28
1.9  Associated conditions ................................................................. 29
1.9.1  Kaposi sarcoma ................................................................. 29
1.9.2  Non-Hodgkin lymphoma ....................................................... 31
1.9.3  POEMS and TAFRO ............................................................ 32
1.10  Treatment modalities ............................................................... 33
1.10.1  Unicentric disease ............................................................. 33
1.10.2  Multicentric disease ........................................................... 33
1.11  Study justification ................................................................. 35
1.12  Aims and Objectives ............................................................. 36

CHAPTER 2: ....................................................................................... 37

MATERIALS AND METHODS .......................................................... 37
2.1  Ethics approval ........................................................................ 37
2.2  Study design ........................................................................... 37
2.3  Sample selection ...................................................................... 37
2.4  Morphological analysis .......................................................... 38
2.5  Immunohistochemistry ............................................................. 39
2.5.1  Antibodies ........................................................................ 39
2.6  Immunohistochemical methods .............................................. 40
2.6.1  Immunohistochemistry – Single antibody method ............... 40
2.6.2  Envision G/2 DoubleStain System (DAKO) Method (Table 2) . 41
2.7  Dual immunohistochemical staining profiles .......................... 42
2.8  Immunohistochemical analysis .............................................. 43
2.9  Statistical analysis ................................................................ 43

CHAPTER 3: ....................................................................................... 45

RESULTS ......................................................................................... 45
3.1  Study cases ............................................................................ 45
3.2  Morphological analysis .......................................................... 45
3.3  Age ....................................................................................... 50
3.4  Sex distribution ...................................................................... 51
3.5  Race ...................................................................................... 52
3.6  Lymph node status ............................................................... 52
3.7  HHV-8 status ...................................................................... 52
3.8  HIV status ........................................................................... 53
3.9  CD4 counts and HAART ......................................................... 54
3.10  Concurrent Kaposi sarcoma ................................................... 56

viii
3.11 Localisation of DC-SIGN and DC-SIGNR within lymph nodes.......................57
3.12 Localisation of p24 within lymph nodes..................................................58
3.13 Location and density of LANA-1 positive cells amongst CD subtypes ..........59
3.14 LANA-1/DC-SIGN and LANA-1/DC-SIGNR............................................62
3.15 LANA-1 and p24.........................................................................................65
3.16 LANA-1 and CD20......................................................................................68
3.17 LANA-1 and CD3.........................................................................................68
3.18 LANA-1 negative CD....................................................................................68

CHAPTER 4: .................................................................................................70
DISCUSSION .................................................................................................70
4.1 Incidence and prevalence ..............................................................................71
4.2 Patient demographics....................................................................................72
4.3 Anatomical location ......................................................................................73
4.4 Unicentric and Multicentric CD....................................................................74
4.5 HHV-8 positive CD ......................................................................................75
4.5.1 HIV status ................................................................................................77
4.5.2 Co-existent Kaposi Sarcoma .......................................................................79
4.5.3 Dual staining LANA-1 and DC SIGN/DC-SIGNR ........................................81
4.5.4 Dual staining LANA-1 and p24 ..................................................................84
4.5.5 Dual staining LANA-1 and CD20 ...............................................................87
4.5.6 Dual staining LANA-1 and CD3 ..................................................................89
4.6 HHV-8 negative cases ..................................................................................89
4.7 Study limitations ..........................................................................................90

CHAPTER 5: .................................................................................................92
CONCLUSION AND FUTURE RESEARCH ..................................................92
5.1 Conclusion ....................................................................................................92
5.2 Future Research ..........................................................................................93
REFERENCES ..................................................................................................96
APPENDIX 1: DATA COLLECTION TEMPLATE ..............................................112
APPENDIX 2: ETHICS PROTOCOL APPROVAL .............................................113
LIST OF FIGURES

Figure 1: HHV-8 interaction with B-cell populations ..........................................................24
Figure 2: Structure of DC-SIGN ..........................................................................................25
Figure 3: Number of cases of CD diagnosed at GSH from 2003 to 2013 (Jan-Jul) ...........45
Figure 4: Castleman disease subtypes .................................................................................46
Figure 5: Density of LANA-1 staining .................................................................................47
Figure 6: Number of CD subtypes originally diagnosed (per year) ..................................48
Figure 7: Number of CD subtypes after morphological reclassification (per year) ........48
Figure 8: Breakdown of original and reclassified CD subtypes .......................................49
Figure 9: Number of CD subtypes before and after morphological reclassification ....50
Figure 10: Age distribution amongst CD subtypes ............................................................51
Figure 11: LANA-1 immunohistochemistry results summary ........................................52
Figure 12: Comparison of CD4 counts amongst subtypes ................................................55
Figure 13: Castleman disease and Kaposi sarcoma .........................................................56
Figure 14: Distribution of diagnosed CD subtypes with concurrent KS ............................57
Figure 15: Lymph node localisation of DC-SIGN and DC-SIGNR ..................................58
Figure 16: Localisation of p24 within germinal centres and mantle zones .....................59
Figure 17: Density of LANA-1 cells ....................................................................................60
Figure 18: Comparison of LANA-1 density amongst CD subtypes .................................61
Figure 19: Co-localisation of LANA-1 and DC-SIGN/ DC-SIGNR within lymph nodes .62
Figure 20: Co-staining LANA-1/DC-SIGN (A) and DC-SIGNR .......................................64
Figure 21: Comparison of co-staining LANA-1/DC-SIGN cells amongst CD subtypes ...65
Figure 22: Double immunohistochemical staining with LANA-1 and p24 ......................66
Figure 23: Comparison of co-staining LANA-1 and p24 cells amongst CD subtypes ......67
Figure 24: Co-staining LANA-1 and CD20 in Pb CD ......................................................68
Figure 25: LANA-1 negative CD .......................................................................................69
LIST OF TABLES

Table 1: Primary antibodies ...........................................................................................................39
Table 2: Kits used for the IHC protocol .........................................................................................40
Table 3: Localisation of double antibody staining .........................................................................42
Table 4: Overview of reclassified CD cases ....................................................................................49
Table 5: Male:Female ratio amongst reclassified CD subtypes .......................................................51
Table 6: HIV and HHV-8 distribution of cohort .............................................................................53
Table 7: Comparison of CD type with Age and Sex .......................................................................54
Table 8: Density of LANA-1 cells .....................................................................................................61
Table 9: Number of LANA-1/DC-SIGN co-staining cells amongst CD types ...............................62
Table 10: Number of LANA-1/ DC-SIGNR co-staining cells amongst CD types .........................63
Table 11: Number of co-expressing LANA-1 and p24 cells ............................................................67
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>CHBH</td>
<td>Chris Hani Baragwanath Academic Hospital</td>
</tr>
<tr>
<td>CD</td>
<td>Castleman Disease</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate recognition domain</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DC-SIGNR</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin related protein</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FLICE</td>
<td>FADD-like interleukin-1-beta-converting enzyme</td>
</tr>
<tr>
<td>GSH</td>
<td>Groote Schuur Hospital</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HHV-8</td>
<td>Human herpes virus 8</td>
</tr>
<tr>
<td>hIL-6</td>
<td>Human interleukin 6</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HIV-ELISA</td>
<td>Human immunodeficiency virus enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HPF</td>
<td>High power field</td>
</tr>
<tr>
<td>HV-CD</td>
<td>Hyaline vascular Castleman Disease</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>iMCD</td>
<td>Idiopathic multicentric Castleman Disease</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi sarcoma</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi sarcoma Herpes virus</td>
</tr>
<tr>
<td>LANA-1</td>
<td>Latency associated nuclear antigen 1</td>
</tr>
<tr>
<td>LIS</td>
<td>Laboratory information system</td>
</tr>
<tr>
<td>MDDC</td>
<td>Monocyte derived dendritic cell</td>
</tr>
<tr>
<td>MCD</td>
<td>Multicentric Castleman Disease</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>Pb-CD</td>
<td>Plasmablastic Castleman Disease</td>
</tr>
<tr>
<td>PC-CD</td>
<td>Plasma cell Castleman Disease</td>
</tr>
<tr>
<td>PEL</td>
<td>Primary effusion lymphoma</td>
</tr>
<tr>
<td>POEMS</td>
<td>Polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes</td>
</tr>
<tr>
<td>SA</td>
<td>South Africa</td>
</tr>
</tbody>
</table>
SMDC  Submucosal dermal dendritic cell
SSA   Sub-Saharan Africa
TAFRO Thrombocytopenia-anasarca-fever-renal insufficiency-organomegaly
UCD   Unicentric Castleman Disease
UK    United Kingdom
USA   United States of America
vCYC  Viral cyclin
VEGF  Vascular endothelial growth factor
vFLIP Viral FLICE-inhibitory protein
vIL-6 Viral interleukin 6
WHO   World Health Organisation
CHAPTER 1:
LITERATURE REVIEW

1.1 Introduction
Castleman disease (CD), previously known as giant lymph node hyperplasia, lymphoid hamartoma and angiofollicular hyperplasia; is a heterogenous, non-clonal lymphoproliferative disorder often accompanied by a constellation of clinical features and a variety of constitutional symptoms.

The observation that Castleman disease could arise in more than one region, led to the first clinical classification system for the disease. Clinically it can manifest in a localised form referred to as unicentric Castleman Disease (UCD) with mild symptoms; or in a multicentric form referred to as multicentric Castleman disease (MCD) characterised by a myriad of systemic symptoms and abnormal laboratory investigations.

The main driver for the dysregulation of the immune system in either form is viral interleukin-6 (vIL-6), aetiologically linked to Herpes Human virus 8 (HHV-8)/Kaposi Sarcoma Herpes Virus (KSHV) (El-Osta & Kurzrock, 2011). Cases of CD not showing any demonstrable infection with HHV-8 (serologically or immunohistochemically) are currently referred to as idiopathic MCD (iMCD) (Fajgenbaum et al., 2016). Although these cases frequently display similar immunological dysfunction observed in viral related cases, the associated hyperactivation of the immune system is mainly due to human interleukin-6 (hIL-6) elaboration (Fajgenbaum et al., 2014; Liu et al., 2016).

Since its initial description, the histopathological classification has also expanded, with the four commonly recognised histopathological subtypes of CD: hyaline vascular (HV-CD), plasma cell (PC-CD), mixed, and more recently the plasmablastic (Pb-CD) subtype.

Pb-CD lymph node changes are only seen in cases of HHV-8-associated MCD. Previously associated with severe immunosuppression, it is now being encountered with increasing frequency in HIV infected patients, regardless of HAART or CD4 counts (Mylona et al., 2008; Powles et al., 2009).
The 2016 South African HIV adult prevalence (15–49 years) in South Africa is 18.9 %, which translates into an estimated 7.1 million individuals infected with HIV. South Africa also has the largest ART programme worldwide with more than 3.6 million people on HAART. The life expectancy of people living with HIV has significantly increased from 53.4 years in 2004 to 62.5 years in 2015, which is accompanied by increasing incidences of co-morbidity, specifically lymphoproliferative disorders (Statistics South Africa, 2016). The reported seroprevalence of HHV-8 in South Africa is 44%-48% (Malope et al., 2008; Maskew et al., 2011). These statistics are noteworthy as the oncogenic potential of HHV-8 is known to dramatically elevate the risk for lymphoid malignancies occurring within immunodeficient populations. Furthermore, HIV has recently been shown to also have oncogenic potential.

Our hospital complex (GSH) provides health care services to a large population of patients in Cape Town metropole and surrounds. Although the province has South Africa’s lowest HIV prevalence rates, it has one of the highest HHV-8 seroprevalence rates. This, in combination with migratory factors most likely play important roles in the increased incidence of HIV/HHV-8 associated CD diagnosed in GSH. Migratory factors may indeed contribute to shaping the demographic of the disease in our hospital setting as the Western Cape is also known to have one of the highest migration streams in South Africa (Statistics South Africa, 2016).

This is pertinent as sub-Saharan Africa has a disproportionately high HHV-8 seroprevalence rate. The reason for the increased risk of the disease while on HAART has not been clearly defined and current knowledge of the pathophysiology of HIV related disease is still limited. The study objectives were primarily to determine the presence and role of DC-SIGN presence in HHV-8 infected cells, determine whether HHV-8 and p24 (HIV) co-infection occurs in the same cells and to determine whether HHV-8 infects B and/or T cells so as to gain further insight into the pathogenesis of Castleman disease. The rarity of the disease underlies the paucity of studies emerging from the South African context however it is becoming increasingly apparent that we need to improve our current knowledge of CD, especially HIV-associated CD. Documentation of the various
demographic aspects of the disease will be of historical, clinical and pathological interest as the long term effects of highly active antiretroviral therapy (HAART) begin to emerge.

It is our hope that our findings contribute to the expanding body of work generated by this intriguing disease, improve understanding of the biochemical intricacies of the co-infected HIV-HHV-8 microenvironment and ultimately generate new information leading to additional advances in early therapeutic and preventative strategies for HIV positive patients at risk for HHV-8 related disease.

1.2 Historical perspective
Castleman disease was first described by Dr Benjamin Castleman, a pathologist at Massachusetts General Hospital, in a 1954 case report of two patients and again in 1956 in a case series of 13 patients (Castleman & Towne, 1954; Castleman et al., 1956). The histological descriptors currently in use were first described in the late 1960s and early 1970s. It was around this time that Flendrig and Schilling (1969) observed that some lymph nodes of patients with the disease were enriched with plasma cell infiltrates rather than the prominent vascular hyalinisation previously described. Keller et al. (1972) were amongst the first researchers who used the terms hyaline-vascular (HV) and plasma-cell (PC) to describe the two main recognised subtypes of CD. They also further refined the characterisation of the plasma-cell subtype, which although less common, was more frequently associated with systemic signs and symptoms. They observed that the involved lymph nodes in this subtype differed little from normal lymph node histology and did not show evidence of prominent vascular hyalinization. The patients with this subtype often presented with generalised lymphadenopathy and were more likely to have a protracted clinical course and systemic B-symptoms, which included anaemia, pancytopenia and hypergammaglobulinaemia. As a result of these very specific findings, the first case of MCD was reported in 1978 while the salient differences between hyaline-vascular and plasma-cell subtypes were further refined (Gaba et al., 1978).

The observation of synchronous Kaposi sarcoma (KS) and MCD was first described in 1985 (Lachant et al., 1985). It was only when Chang and associates discovered a novel Gamma-Herpes virus in 1994, which was subsequently named Human Herpesvirus 8 (HHV-8)/Kaposi sarcoma Herpes virus (KSHV), that the first cases of HHV-8 associated MCD were described (Chang et al., 1994; Cesarman et al., 1995). Following this, a causal
link between HHV-8 and KS was then identified in both HIV-positive and HIV-negative patients in the mid 1990s (Moore and Chang, 1998). Soulier et al. (1995a) then reported on the presence HHV-8 sequences in the lymph nodes of both HIV-positive and HIV-negative patients with MCD. Other investigators later confirmed that virtually all HIV-positive CD cases contained HHV-8 in their affected lymph nodes and that only half of HIV-negative MCD were HHV-8 positive (Dispenzieri et al., 2012). Detection of HHV-8 LANA-1 has therefore been crucial in the diagnosis of HHV-8 associated cases. Consequently, the absence of HHV-8 almost always excludes HIV related disease (Marcelin et al., 2003). Yoshizaki et al. (1989) were the first researchers to report the elevated levels of interleukin 6 (IL-6) by B-lymphocytes in the germinal centres in CD lymph nodes. This novel discovery eventually led to the development of anti-IL6 and anti-IL6R monoclonal antibodies as treatment options for CD (Beck et al., 1994; Nishimoto et al., 2000; van Rhee et al., 2015). The identification of the additional elaboration of the vIL-6, which is primarily encoded by HHV-8 in both MCD and KS, further cemented the relationship between these two diseases (Aoki et al., 1999).

1.3 Classification

1.3.1 Current classification

Historically, the classification of CD has been based on both clinical and radiological parameters at the time of diagnosis. These findings usually distinguish two distinct entities that usually correspond with relevant symptomatology: being either UCD or MCD at presentation. Histologically, the disease is further subdivided into four subtypes: hyaline-vascular (HV), plasma-cell (PC), mixed and the more recently described plasmablastic subtype, which is associated with more aggressive disease and seen only in HIV/HHV-8-associated cases (Cronin & Warnke, 2009). It remains controversial as to whether CD subtypes represent different stages of the same disease or whether they are entirely separate disease entities. Even as early as the 1970s, when the disease was first being described, there was recognition that both UCD and MCD were often part of a continuous spectrum that ranged from unifocal, unicentric disease to multicentric disease which Keller et al (1972) claimed reflected the evolution of CD with time. This view was validated by reports of transitions between HV and PC subtypes on follow-up biopsies of affected patients as well as by the simultaneous presence of both subtypes at separate sites within the same patient (Keller et al., 1972).
The frequent finding of co-existent HV- and PC-CD (mixed subtype) in the same lymph node, especially in the setting of HHV-8 infection, strongly favours the argument of a single disease with variable histopathology during the evolution of the disease. As there are no official diagnostic criteria for HIV/HHV-8 associated CD or specific laboratory tests to confirm its presence, it is important that a surgically excised lymph node be histologically and immunohistochemically evaluated once the diagnosis is suspected. This not only helps to render the correct diagnosis but also helps to exclude reactive states, autoimmune diseases and malignancies that can cause atypical lymph node hyperplasia with overlapping histopathological features (Soma & Kara, 2014).

1.3.2 Proposed classification

Until very recently, the classification of CD has been distinguished by HIV status alone. The frequent identification of HIV-negative, HHV-8 negative disease led some investigators to suggest that the HIV-based classification system was inadequate as it did not distinguish all the possible driving factors of CD pathogenesis (Ide et al., 2006; Naresh et al., 2009). The newly proposed classification system states that the diagnosis of CD made primarily on the HHV-8 status is more appropriate, as it is thought to be more appropriate since it is linked to pathogenesis and treatment responses (Fajgenbaum et al., 2014; Liu et al., 2016).

1.4 Pathology

1.4.1 Hyaline vascular subtype

HV-CD is characterised by lymphoid proliferation with frequently regressed lymphoid follicles, small atrophic germinal centres and widened mantle zones with small lymphocytes arranged concentrically in an “onion-skin” fashion (McCarty et al., 1995; Kojima et al., 2008). The appearance of a single hyalinized blood vessel penetrating the follicle together with the concentric rimming by mantle zone lymphocytes is often described as resembling a ‘lollipop’ (Lin & Frizzera, 1997). The interfollicular regions are often expanded with prominent hyalinised blood vessels, admixed myoid cells, dendritic reticulum cells and T- lymphocytes (Nyugen et al., 1994; Cronin & Warnke, 2009). In addition, focally enlarged dysplastic follicular dendritic cell (FDC) networks are present. These follicular dendritic cells can sometimes be seen in aggregates within these expanded interfollicular zones (Harris & Bhan, 1987; Jegalian et al., 2009). There is usually no observable increase in plasma cell infiltrates or eosinophilic aggregates and the lymph
node sinuses are often attenuated or closed (Nyugen et al., 1994). The hypervascularity observed in HV-CD is thought to be driven by increased intrafollicular vascular endothelial growth factor (VEGF) expression (Bates & Harper, 2002). Alterations in the follicular dendritic cell networks can eventually lead to neoplastic proliferations of dysplastic follicular dendritic cells with the eventual formation of follicular dendritic cell sarcoma, which is a rare malignancy that occurs more commonly in lymph nodes with HV-UCD (Chan et al., 1994; Cronin & Warnke, 2009). This malignancy may also involve extranodal sites and can either be diagnosed concurrently with or following the diagnosis of UCD (Chan et al., 1997; Lin & Frizzera, 1997).

### 1.4.2 Plasma cell subtype

Although the lymph nodes in PC-CD show less distinctive features than those seen in HV-CD, there is usually preserved architecture with hyperplastic, rather than atrophic, lymphoid follicles (Nyugen et al., 1994). These follicles contain larger and more active germinal centres that are surrounded by narrower mantles of mature lymphocytes with no associated expansion or abnormality of the FDC network (Cronin & Warnke, 2009). The most striking feature of this subtype is the noticeable increase in interfollicular plasma cell infiltrates which are usually polyclonal in nature (Menke & DeWald, 2001). These plasma cells may rarely be monoclonal, for example, in osteosclerotic myeloma, with associated λ light chain restriction (IgG or IgA) (Hall et al., 2006) and present with POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and/or skin changes) (Ohyashiki et al., 1994; Al-Maghrabi et al., 2006). PC-CD like changes can also be seen in both reactive lymphadenopathies which include infections, autoimmune diseases, collagen vascular and mixed connective tissue diseases as well as in immunodeficiency-related lymphadenopathies (McClain et al., 2004; De Marchi et al., 2004). The multicentric form is almost exclusively of the plasma cell subtype and is frequently encountered in the immunosuppressed HIV-positive patient (Shahidi et al., 1995; Cronin & Warnke, 2009). It is often described in association with POEMS syndrome (polyneuropathy, organomegaly, monoclonal endocrinopathy, gammopathy and skin changes) (Ide et al., 2006). The unicentric form of PC-CD is probably the least well-characterised subtype with many cases most likely representing a localised presentation of multicentric plasma cell CD (Cronin & Warnke, 2009).
1.4.3 Plasmablastic subtype

Another unique subtype exclusively associated with HIV is the plasmablastic subtype (Dupin et al., 2000; Ide et al., 2006; Naresh et al., 2009). It is reported to be the most common subtype observed during HIV infection (Powles et al., 2007). Most studies looking for viral particle sequences in these cases of HIV-MCD have shown high rates of HHV-8 infection (Stebbing et al., 2008; Naresh et al., 2008). When localised, this type usually affects an aggregated mass of lymph nodes (Dupin et al., 2000). It characteristically shows both plasma cell infiltrates in conjunction with an associated population of larger plasmablasts, the majority of which localise to the outer follicular mantle zones and paracortical regions.

These plasmablasts are characterized by a moderate amount of amphophilic cytoplasm and large vesicular nuclei with 1–2 prominent nucleoli. They express OCT2, BLIMP1, and IRF4/MUM1, but lack positivity for PAX5, BCL6, CD138, and EBER (Dupin et al., 2000). The plasmablasts express high levels of cytoplasmic IgM with monotypic λ light-chain expression, but show a polyclonal pattern of immunoglobulin gene rearrangement (Du et al., 2001; Chadburn et al., 2008). They can even show weak, variable expression of CD20 (Dupin et al., 1999; Chadburn et al., 2008; Naresh et al., 2009).

HHV-8 appears to also target IgM monotypic lymphocytes in the mantle zone of lymphoid follicles, which do not have somatic hypermutation of IgH chain genes, which indicates that these cells are derived from naïve B-lymphocytes. These cells are often immature B-cells that have not undergone the germinal centre activation required for normal B-lymphocyte maturation (Du et al., 2001). Dupin et al. (2000) have also demonstrated that HHV-8 is also able to infect B cells with plasmacytic differentiation, in both the mantle zones as well as in the interfollicular regions. As the majority of these cells are HHV-8 infected, immunohistochemical staining with the monoclonal antibody directed against HHV-8 LANA-1, can be used to confirm these virally infected plasmablasts (Parravicini et al., 1997; Dupin et al., 2000; El-Osta & Kurzrock, 2011).

Schulz (2006) demonstrated that up to 50% of the B cells within the mantle zones of the affected lymph node can show positive LANA-1 staining. Although these plasmablasts mainly aggregate within the outer follicular mantle zones, they can eventually colonize the germinal centres (Cronin & Warnke, 2009). Over time and within a background of
immunosuppression and HHV-8 infection, these plasmablasts can undergo clonal expansion with the resultant development of “microlymphomas” (Dupin et al., 2000; Stebbing et al., 2008). These lesions can eventually progress to a plasmablastic lymphoma (Li et al., 2006). vIL-6, encoded by HHV-8 and also expressed in the infected plasmablasts, is responsible for driving malignant lymphoproliferation (Parravicini et al., 1997; Aoki et al., 1999). HHV-8-positive plasmablastic lymphoma is now regarded as a distinct entity arising in a background of HHV-8-associated MCD (Cronin & Warnke, 2009). These lymphomas are distinguished from other classic plasmablastic lymphomas arising in other settings due to the fact that the neoplastic cell population is genetically distinct, having arisen from naïve, IgM-producing plasma cells without immunoglobulin hypermutation (Dupin et al., 2000; Li et al., 2006).

Du et al. also demonstrated that plasmablasts coinfected with both HHV-8 and EBV can sometimes exist in lymph nodes showing morphologically similar features to HHV-8-associated MCD (Du et al., 2001). These rare cases were subsequently referred to as ‘Kaposi Sarcoma-associated Herpes Virus-associated Germinotropic Lymphoproliferative Disorder’ (Cronin & Warnke, 2009).

1.4.4 Mixed subtype
The mixed subtype of CD shares morphological features of the hyaline vascular subtype and plasma cell subtype. It is classically encountered in MCD and is typically seen in HIV-associated MCD (Gaba et al., 1978; Weisenburger et al., 1985).

1.4.5 Idiopathic multicentric Castleman Disease
Although the role of HHV-8 in the pathogenesis of HIV-positive MCD is clearly defined, its role, if any, in HIV-negative disease remains controversial and for many cases of MCD that are negative for HHV-8, the aetiology still remains unclear. As a result, these HIV-negative, HHV-8 negative cases have been collectively classified as idiopathic MCD (iMCD) (Fajgenbaum et al., 2014; Liu et al., 2016).

As iMCD patients also exhibit high cytokine levels, with IL-6 playing a central pathogenic role, Fajgenbaum et al. (2014) propose that iMCD most likely represents a common endpoint for multiple immunological processes that primarily involves immune dysregulation. Some of the postulated mechanisms for iMCD include autoinflammatory
hypercytokinaemia generated by autoantibodies, abnormal cytokine secretion by affected cells within the lymph nodes or viral signalling by non-HHV-8 viruses (Fajgenbaum et al., 2014).

1.4.5.1 Clinical and pathological features of iMCD

In the past, the diagnosis was made primarily on “Castleman lymph node histopathological features” combined with thorough clinical correlation that excluded all other infectious, autoimmune and neoplastic diseases (Fajgenbaum et al., 2014).

The proposal is that iMCD cases should have negative HIV serology, show no detectable replicating HHV-8 virus in the peripheral blood and have negative staining for LANA-1 in lymph node tissue (Fajgenbaum et al., 2014). The absence of LANA-1 is key to the diagnosis Diagnostic criteria by the French Agence Nationale de Recherches sur le SIDA (ANRS) and the National Cancer Institute (NCI) proposed for patients with iMCD proved to be helpful (Gerard et al., 2007; Uldrick et al., 2012). This is due to the fact that histopathological diagnosis in HIV/HHV-8 associated MCD is made much easier by the complementary use of DNA tests to detect the presence of HHV-8 in the peripheral blood and positive LANA-1 immunostaining (Bower, 2010). Like HHV-8 associated MCD, iMCD can also show features of both hyaline vascular and plasma cell subtypes. However, iMCD generally presents with marked plasmacytosis and a lesser degree of vascular proliferation and hyalinisation, in comparison to HHV-8 associated MCD (Suda et al., 2001).

There is often significant overlap with malignant, autoimmune, and infectious disorders which makes accurate diagnosis challenging. Until recently there were no standardised diagnostic criteria or diagnostic biomarkers. Fajgenbaum et al. (2016) published seminal consensus criteria for Castleman Disease diagnosis, with special reference to iMCD cases. These include major criteria (characteristic lymph node histopathology and multicentric lymphadenopathy), at least 2 of 11 minor criteria with at least 1 laboratory abnormality. Importantly, infectious, malignant, and autoimmune disorders that can mimic iMCD need to be excluded.
1.5 Epidemiology of unicentric (UCD) and multicentric (MCD)

Since the discovery of HIV over three decades ago, the prevalence of CD has progressively increased. The prevalence in the United States was estimated to be less than 1/100,000 (Degot et al., 2009). Recent estimates suggest the incidence of all subtypes of CD (UCD, HHV-8-associated MCD and iMCD) to be approximately 6,500 to 7,700 new cases per year in the USA (Munshi et al., 2015). The global incidence of MCD is 4.3/10,000 patient-years (Oksenhendler et al., 2002; Powles et al., 2009). There is no observable racial or ethnic predilection (Aguilar-Rodriguez et al., 2014). There have been no formal studies with regards to incidence or prevalence of CD in South Africa. There have, however been a few case reports and small case series from centres in South Africa, such as a series of 35 adult patients with MCD, who were seen over a twenty-five year period at Chris Hani Baragwanath hospital (Patel et al., 2015). The average survival for UCD is greater than 10 years with an overall 5-year survival rate of 90%. The mortality rate of MCD approaches 35% and is worse than both prostate and breast cancer (Munshi et al., 2015). The 5-year overall survival was 65% in 2012 series of MCD cases, while the 3-year disease-free survival was reported at 46% (Talat & Schulte, 2011; Dispenzieri et al., 2012).

CD is very rarely reported in children and is predominantly diagnosed during the teenage years with a slight predilection of girls (Parez et al., 1999). The majority of these childhood cases belong to the hyaline vascular type (Soumerai et al., 2014), which usually presents in a unicentric manner with a relatively asymptomatic course, the majority of symptoms being due to local mass effect. There are only about 100 paediatric cases published. MCD is rare in children with only few cases reported to date, but follows a more favourable course compared to adults (Parez et al., 1999). Leroy et al. (2012) conducted a systematic literature review and found that almost half of the cases of paediatric MCD originated from areas in which HHV-8 is endemic.

The classic HV-CD usually presents unicentrically and is found in 90% of UCD (Waterston & Bower, 2004; Collins et al., 2006). It presents as a localised mass, most frequently occurring in a lymph node although extranodal presentations can also occur (Cronin & Warnke, 2009). There is usually an absence of systemic signs and symptoms which are more commonly associated with MCD (Dispenzieri et al., 2012). UCD most often presents in the third and fourth decades of life with the average age at diagnosis
being 34 years (range: 2–84 years) (Soumerai et al., 2014). Although some studies have shown that there is a slight female predominance (1.4:1) (Talat et al., 2012), other series have shown no sex predilection (Keller et al., 1972; Herrada et al., 1998). There is often no identifiable association with HIV or HHV-8 infection and definite epidemiological risk factors have not been established (Mylona et al., 2008). If it is seen in the HIV-infected patient, it usually also co-exists with the plasma cell subtype, either within the same node or in a different node (Stebbing et al., 2008).

Unicentric PC-CD accounts for 9% to 24% of localised CD (Keller et al., 1972; Frizerra, 1988; Menke et al., 1996) and 80-90% of MCD (Waterston & Bower, 2004; Collins et al., 2006). Localised PC-CD shows a relatively similar sex and age distribution to the hyaline vascular subtype (Frizerra, 1988; Menke et al., 1996; Cronin & Warnke, 2009). Clinically, PC-UCD usually involves an aggregated mass of enlarged nodes as opposed to a dominant single node involved in HV-CD (Cronin & Warnke, 2009). However even when localised, the unicentric plasma cell subtype is more likely to be associated with increased IL-6 serum levels, abnormal laboratory findings and systemic symptoms documented in the multicentric presentations (Flendrig & Schillings, 1969; Frizerra, 1988; Menke et al., 1996; Casper, 2005). The difference, though, is that unlike multicentric PC-CD which requires systemic therapy, surgical excision is reportedly curative in unicentric PC-CD (Brandt et al., 1990). Multicentric PC-CD has been reported to present in the sixth decade of life (Frizzera et al., 1985; Herrada et al., 1998). Patients with HIV-associated MCD (both plasma cell and plasmablastic subtypes) however, although showing no age or sex predilection, tend to present at younger age with a slight male predominance (Al-Natour et al., 2010; Bower et al., 2011., Soumerai et al., 2014).

Studies have also revealed an increased incidence of HIV-associated MCD since the introduction of antiretroviral therapy (Mylona et al., 2008; Soumerai et al., 2014). The exact mechanism for this is unclear. It may be attributed to longstanding immune dysregulation associated with long-term HIV infection, improved survival rates or even an increased awareness of the disease among treating health care professionals (Soumerai et al., 2014). In contrast, there is a decreasing incidence of Kaposi sarcoma (KS) observed in patients enrolled on HAART (Powles et al., 2009).
1.6 Pathogenesis of CD

Current knowledge regarding the aetiopathogenesis of CD is based largely on case series and histopathologic reviews accumulated over the last decade. Clinical and laboratory investigations of patients with the different CD subtypes have enabled a greater understanding of the pathophysiology and management of CD. Although CD was discovered more than fifty years ago, its exact pathophysiologic basis had not been fully elucidated. The valuable information gathered from both clinical and pathological series of patients with CD led early investigators to identify elevated levels of IL-6 often driven by HHV-8, in the absence of other initiating factors. The abnormally high levels of IL-6 serves as the triggering factor for the initiation of pathological processes within a defective immune system, eventually leading to lymphoid proliferation, angiogenesis and systemic manifestations characteristic of MCD. Plasmablastic MCD was rarely identified early in the HIV pandemic and it received little attention until the finding of HHV-8 infected plasmablasts (Dupin et al., 1999; Dupin et al., 2000).

1.6.1 Human Herpes virus 8

Early studies initially focused on isolating EBV-DNA within lymph nodes from patients diagnosed with CD to determine if EBV was the causative agent (Castleman et al., 1956; Keller et al., 1972). Despite occasional CD cases showing EBV-DNA, its rare presence did not prove causation and these small studies eventually argued against EBV as the aetiological agent (Murray et al., 1995). The 1994 discovery of the gamma-herpesvirus in KS tissue samples had an enormous impact on virology as it was found to be the causative factor for all forms of KS (Chang et al., 1994). As KS had also been frequently reported in patients with MCD, these findings led researchers to investigate the link between HHV-8 infection and CD (Soulier et al., 1995a/b).

Since HIV is also highly replicated within lymphoid tissue, it was also considered to potentially play a role in the pathogenesis of the disease (Collins et al., 2006). The pathogenesis of HIV-associated CD was then found to involve an interplay between the two viruses, HHV-8 and HIV, cytokines such as IL-6 and growth factors such as VEGF (Oksenhendler et al., 2000). The relationship between HHV-8 and MCD has been extensively studied in the context of HIV infection (Soulier et al., 1995a). Soulier et al. (1995b) also demonstrated that HHV-8 sequences were detected in lymph nodes in 100% of cases of HIV-associated MCD, compared to only 41% of cases with HIV-negative
MCD while other studies have confirmed an almost universal association of HHV-8 with HIV-associated MCD (Bower et al., 2011). The overall conclusion of these studies was that HHV-8 negative CD did not exist in HIV-positive patients.

1.6.1.1 **History of HHV-8**
Before the discovery of HHV-8 using molecular techniques almost all viruses had been identified using conventional virus isolation methods with cell cultures. Chang et al. (1994) identified DNA fragments of the virus in KS tissues and used a representational difference assay, a subtraction PCR-based method, to show the presence of these unique herpes viral DNA sequences. HHV-8 thus, became the first virus whose fragments were identified directly by the PCR method before any cell culture methods.

When KS was identified in 1872 by Moritz Kaposi, it was considered a rare cancer, affecting mostly elderly men of Mediterranean descent (termed Classical KS) (Kaposi, 1872). In the early to mid-1980s, new cases of KS were observed in young men who showed none of the previous risk factors for KS and were later found to be AIDS patients. At the time it was not understood whether HIV was involved in the development of KS but the cancer was listed as one of the defining illnesses of AIDS. It was only after the findings of Chang and associates that it was understood that KS resulted from a co-infection in these patients with both HIV and HHV-8 (Chang et al., 1994). Since then HHV-8 has also been identified in transplantation related KS (Hengge et al., 2002a). Over the 23 years since the discovery of HHV-8, it has been established as an oncogenic virus with lymphotrophic, oncogenic properties. The oncogenic properties of HHV-8 and the fact that it has been aetiologically linked to two B cell malignancies, primary effusion lymphoma (PEL) and HHV-8+ plasmablastic lymphoma (Hengge et al., 2002b), has led to its classification as a class I carcinogen by the International Agency for Research on Cancer (IARC) (Gramolelli & Schulz, 2015).

1.6.1.2 **Epidemiology and molecular characteristics of HHV-8**
Epidemiological studies suggest that HHV-8 has existed in the human population for at least a few centuries (Dukers & Rezza, 2003) and serological studies have revealed that HHV-8 infected individuals are found all over the world with seroprevalence of infection differing according to regions and countries. More than 50% of HHV-8 seropositivity is
found in sub-Saharan Africa, 10-30% in Mediterranean regions and less than 10% in America, Asia and Northern Europe (Katano et al., 2000a).

Phylogenetic analysis has revealed several distinct subtypes, which are believed to have diverged at least 10 000 years ago. HHV-8 is the most recently identified human oncogenic herpesvirus expressing viral oncogenes which constitutively activate growth-signalling pathways (Hengge et al., 2002a; Verma & Robertson, 2003). HHV-8 is most closely related to Rhesus Rhadinovirus (RRV), while its nearest human herpesvirus relative is Epstein-Barr virus (EBV, HHV-4) (Alba et al., 2001). Like other herpesviruses, the HHV-8 genome consists of linear, double-stranded DNA (170 kbp) with a long unique region (LUR) and terminal repeats (TRs) at both termini (Russo et al., 1996). The HHV-8 LUR is responsible for encoding at least 80 viral proteins and 12 pre-microRNAs (miRNAs) (Cai et al., 2005). These in turn yield at least 25 miRNAs during viral latency, 18 of which are elaborated during this latency period (Liu et al., 2012).

Like other herpesviruses, viral genes are categorized into lytic and latent genes, and also into immediate-early (IE), early (E), and late (L) genes based on their expressions. While all human herpesviruses share considerable homology, with many genes conserved amongst herpesviruses, HHV-8 also contains at least 15 distinct genes unique to itself which have been designated with the prefix “K” (Hengge et al., 2002a). Genotyping of HHV-8 is based on categorising the sequences of the hypervariable regions in its K1 gene (Zong et al., 1999). The HHV-8 K1 is classified into 5 genotypes: A, B, C, D and E with no correlation between genotype and HHV-8 related diseases (Zong et al., 1999).

Some HHV-8-encoded genes are homologous to human oncogenes or cell-cycle associated genes while some are transformational genes, able to transform human cells and destabilise cellular responses to HHV-8 (Russo et al., 1996; Cesarman, 2014). However, the expression of these viral encoded genes is severely restricted with only a few viral genes being expressed in HHV-8 infected cells.

In more than 90% of infected tumour cells, HHV-8 is locked into a latent state and only a subset (1–5%) of the infected cells undergo lytic replication (Verma & Robertson, 2003; Dittmer et al., 1998). Protein expression is therefore restricted to only a few viral genes. The majority of viral proteins are expressed only during active viral replication and only a
few in the early stages of non-productive infections (Russo et al., 1996). Only six protein
products have been shown to be associated with the persistent or latent stage of the viral
life cycle and are encoded by a latency-associated gene cluster. This cluster includes
LANA-1 (Latency-associated nuclear antigen-1, ORF 73), viral cyclin/v-cyclin (ORF72),
viral FLICE-inhibitory protein (K13, v-FLIP), kaposin (K12) and viral interferon
regulatory factor-3 (vIRF-3, LANA-2), and short ORF K11.1 encoding vIRF-2 (Dittmer et
al., 1998).

The viral gene expression profile appears to differ among HHV-8 infected disorders.
Lytic virus expression, including vIL-6, is most common in MCD, to a lesser extent in KS
and relatively uncommon in PEL (Paravicinni et al., 1997; Kaplan, 2013). Recently,
luciferase immunoprecipitation system (LIPS), which is a liquid-phase immunoassay that
uses a light emitting protein to quantitatively measure HHV-8 antibody levels, has been
employed to diagnose infection. One such LIPS study that assesses the serum levels of
latent and lytic HHV-8 antigens, detected significant differences in patients with KS and
MCD (Burbelo et al., 2010).

HHV-8 shares 30–50% homology to EBV and similar to EBV, it can induce latent
infection of peripheral blood lymphocytes, immortalise lymphocytes in vitro, and lead to
the development of malignant lymphomas (Hengge et al., 2002b). Like other
herpesviruses, HHV-8 is also proficient at evading the immune system through its ability
to use viral immunomodulators, many of which are homologues of human genes that
interfere with the host immune response. One of the ways in which HHV-8 evades the
immune system is through encoding of proteins that alter the host immune response from
Th1 to Th2, which is achieved partly by the action of vIL-6, inhibition of complement and
down-regulation of the adaptive immune response (Eaton et al., 2010).

1.6.1.2.1 LANA-1

There are numerous HHV-8 genes that affect binding, cell growth, proliferation,
inflammation and angiogenesis. Most prominent among these genes is LANA-1 which is
involved in episomal maintenance, acts as a transcriptional regulator and modifies
expression of viral and cellular genes (Eaton et al., 2010). LANA-1 transcription is shown
to be under the control of the LANA promoter (LANAp) (Dittmer et al., 1998; Hengge et
al., 2002b). Numerous studies have shown that LANA-1 is a multifunctional protein
always expressed in HHV-8-infected cells both in vivo and in vitro and that latency is maintained in infected cells (Dupin et al., 1999; Kellam et al., 1999). Thus it is evident that LANA-1 plays an important role in the pathogenesis of HHV-8 infection, with the most important function being the ability to establish and maintain latency in infected cells by tethering the viral DNA to the host chromosome via the TR sequences on the HHV-8 genome (Ballestas et al., 1999).

LANA-1 modulates various cellular pathways both positively and negatively to ultimately use the host replicative machinery to drive cell proliferation. LANA-1 is also involved in signal transduction within the affected cell which dysregulates important signalling pathways, especially those involved in oncogenesis (Cesarman, 2014). Viral infection usually results in a protective induction of p53 expression and p53-dependent apoptosis in the cell. Direct binding with LANA-1, however, downregulates the important tumour suppressor pathways regulated by p53 and Rb (Friborg et al., 1999). Binding of LANA-1 to p53, not only inactivates cellular apoptosis, it also blocks the tumour suppressor’s ability to act as a transcriptional activator which allows for viral survival and maintenance of the latent viral episome (Cesarman & Knowles, 1999; Cesarman, 2014). Studies have also demonstrated a role for LANA-1 in regulating HHV-8 reactivation, which involves modulation of the major Notch cellular signalling pathway (Verma & Robertson, 2003). Although LANA-1 is crucial to HHV-8 pathogenesis, it does not have any full transformation activity, which places emphasis on other factors that play a role in HHV-8 oncogenesis.

1.6.1.2.2 vIRF-3/LANA-2
This gene encodes a latent protein that inhibits p53-tumour suppressor pathways and promotes VEGF (Schulz & Cesarman, 2015). It is expressed only in PEL cells and CD but not in KS cells (Rivas et al., 2001).

1.6.1.2.3 Viral cyclin (vCYC)
It is a homologue of cyclin D1 which can inhibit p27/Kip1 and induce the cell-cycle to S-phase (Fukumoto et al., 2011). Viral cyclin is a latent protein that modulates the cell cycle and assists the virus in evading normal regulatory checkpoints which allows the infected cell to overcome cell cycle arrest (Moore et al., 1998).
1.6.1.2.4 vFLIP (ORF K13)

vFLIP, also a latent protein, is the third of the LANA-promoter coding regions that prevents Fas-induced programmed cell death by interfering with the Fas/TNFR pathway and is homologous to the cellular FLICE/caspase 8 inhibitory proteins (cFLIPs), which can inhibit death receptor mediated apoptosis (Kaplan, 2013; Cesarman, 2014). Cell survival is also promoted through vFLIP activation of NFκB (Chaudhary et al., 1999). This causes the activation of numerous cellular genes, including anti-apoptotic genes. In addition, expression of vFLIP and kaposin-B may play a role in enhancing pro-inflammatory cytokine expression and together with VEGF, play an important role in the vascular proliferation seen in KS and CD (Kaplan, 2013).

1.6.1.2.5 Kaposins

Kaposins are encoded in a complex region of the HHV-8 genome that contains the small coding region (K12) which can potentially encode three proteins: Kaposin A, B, and C (Cesarman, 2014). Kaposin B is thought to play a role in stabilisation of cytokine mRNA and may enhance cytokine release (Kaplan, 2013).

1.6.1.3 South African context

Serological studies have revealed that HHV-8 infected individuals are found all over the world (Sitas et al., 1999). The prevalence of HHV-8 infection varies among different geographic areas with higher prevalence in areas where HIV infection is widespread (Caselli et al., 2005).

An estimated 44,247 new cases and 26,974 deaths of KS worldwide was reported in 2012. This data also shows 40,874 of new cases in less developed regions and 3,373 new cases in more developed regions. The African continent is disproportionately affected, where 37,509 (85%) of all cases occur (Globocan: 2012). Malope et al. (2008) reported that up to 48% of HIV-infected adults in South Africa also tested positive for HHV-8. The distribution of HHV-8 infection amongst HIV risk groups mirrors the pattern of KS occurrence in HIV-infected individuals. A high prevalence of KS has been reported in sub-Saharan Africa (Dukers & Rezza, 2003). One of the first and largest studies addressing survival in KS in HIV-infected patients by Bohlius et al. (2014) found that the incidence of KS is significantly lower in patients on established HAART. Mohanlal and Pather (2015) recently conducted a retrospective cross-sectional study of KS patients at
Chris Hani Baragwanath Academic Hospital and reported that the mean age of patients was 37 years, the male/female ratio was 1.2:1 and the median CD4 count was 128 cells/ul. KS remains the most common neoplasm in HIV-infected patients and the relative risk of developing KS in AIDS is much higher than that for other immunosuppressed patient groups (Sitas et al., 2000; Albrecht et al., 2004).

In one South African study, the seroprevalence of HHV-8 was relatively high compared with that in the United States and has been found to increase significantly and steadily from birth into adult life (Sitas et al., 1999). Furthermore, there was lower seroprevalence of HHV-8 in Whites than in Blacks in South Africa.

### 1.6.2 HIV

Immediately after HIV infects a cell, the virion RNA is copied into DNA and the proviral genome is transported into the nucleus for integration into the host cell genome (Aboul-ela et al., 1995). Once integrated into the host chromosomal machinery, the HIV virion is regulated by cellular transcription factors, as well as its own regulatory proteins. HIV transcription is controlled primarily by tat (trans-activator protein), which is one of the first proteins to be expressed after infection occurs. Unlike other transcriptional factors that normally bind DNA proteins, Tat binds RNA proteins and recognises a specific sequence, TAR (Tat activating region), which is present on the HIV-1 RNA molecule that results in increased HIV-1 mRNA production (Frankel & Young, 1998). It is also mainly responsible for elongation by RNA polymerase II. This elongation allows for synthesis of full-length transcripts and increases transcription initiation (Aboul-ela et al., 1995). In the absence of Tat, initiation from the long terminal repeats (LTR) is efficient but transcription is impaired as the promoter engages poorly functioning polymerases that disengage too quickly from the DNA template (Karn and Stoltzfus., 2012). Tat protein is involved in stimulating cellular proliferation, inhibition of apoptosis and also stimulates the expression of growth-promoting cytokines. In addition, Tat has been shown to directly act as an angiogenic factor that can interact with the VEGF-receptor (Hengge et al., 2002b).

#### 1.6.2.1 HIV and HHV-8 interaction

Studies show that the simultaneous intracellular presence of HHV-8 and HIV-1 causes upregulation and reciprocal gene expression (Huang et al., 2001). It has been proposed
that HIV promotes and initiates progressive HHV-8 infection through at least two important paracrine mechanisms (i) by production of the Tat protein and (ii) by promoting cytokine production (Mocroft et al., 1998). HHV-8 may also have a direct effect on HIV infection as demonstrated by the in vitro co-cultivation of HHV-8 infected B-cell lines with HIV-infected CD4 T-cells which resulted in a significant increase of HIV replication (Mercader et al., 2001). Studies also suggest that LANA-1 cooperates with HIV Tat to cause activation of the HIV LTR promoter which supports the proposal that HHV-8 co-infection promotes HIV propagation (Huang et al., 2001). There is also increased susceptibility to HIV in natural targets such as T-lymphocytes as well as otherwise non-permissive cells such as B-lymphocytes, which then allows for transmission of HIV infection to uninfected T-cells (Caselli et al., 2005).

HIV-1 induced immunosuppression also plays an important role in the pathogenesis of HHV-8 associated cancer. HIV infection stimulates HHV-8 replication and reactivation in latently infected cells (Merat et al., 2002). HIV-1 Tat protein can also increase HHV-8-encoded G-protein-coupled receptor signalling, which causes an increase of the HHV-8 lytic biological cycle with accelerated tumourigenesis (Pati et al., 2003; Harrington et al., 1997)

1.6.3 Cytokine factors
1.6.3.1 hIL-6
In the search for a unifying factor in the pathogenesis of CD, IL-6 has emerged as this key factor. Studies have shown that this multifunctional cytokine is central in the pathophysiology of all forms of CD and is closely linked with the deranged systemic manifestations of the disease. The association between the systemic features of CD, especially the plasma cell subtype and a factor secreted by affected lymph nodes was recognized as early as 1989 (Yoshizaki et al., 1989). These investigators also noted an elevated concentration of IL-6 in a patient with UCD, which normalized after lymph node excision. This was also followed by a reduction in clinical and biological abnormalities. This correlates with observations that exacerbations of MCD in HIV-infected patients show high levels of HHV-8 viral load and human IL-6 (Uldrick et al., 2012; Polizzotto et al., 2012; Polizzotto et al., 2013).
IL-6 is a multifunctional, pleiotropic cytokine produced by various cells such as T-cells, B-cells, monocytes, fibroblasts, and endothelial cells (El-Osta & Kurzrock, 2011). It is considered the most important disease mediator in MCD. It binds to a membrane receptor and leads to gp130 dimerization, phosphorylation, and activation of receptor-associated kinases (Rossi et al., 2015). IL-6 has multiple downstream effects that are involved primarily with regulating multiple cellular signalling pathways, mainly via the JAK/STAT pathway (Nishimoto & Kishimoto, 2006), which in turn leads to VEGF expression and signalling (El-Osta & Kurzrock, 2011).

It is also involved in the synthesis of acute-phase reactant proteins by the liver and associated with the development of constitutional symptoms accompanying many inflammatory diseases (Nishimoto & Kishimoto, 2006). Leger-Ravet et al (1991) demonstrated that germinal centres of CD lymph nodes produced large quantities of IL-6 with the subsequent detection of IL-6 secretion by germinal centre B cells. IL-6 has a triple role in the disease process which involves induction and survival of B-cell proliferations, generation of inflammatory symptoms and increased secretion of VEGF resulting in subsequent increased angiogenesis and interfollicular plasma cells, all of which may contribute to the symptomatology observed in CD (Aoki et al., 1999; Nishi et al., 1999).

IL-6 is emerging as both a biologically and prognostically important cytokine in many lymphoid malignancies as it can also function independently as an autocrine or paracrine growth factor in a variety of cancers (El-Osta & Kurzrock, 2011).

1.6.3.2 vIL-6
Apart from germinal centre B-cells, another source of IL-6 production in CD is from HHV-8 infected cells. The virus has been shown to produce a viral analogue of IL-6 (vIL-6) and shares many of the biological activities of hIL-6 (Moore et al., 1996; Parravicini et al., 1997b). Although vIL-6 can activate hIL-6 signalling pathways independently of IL-6R, it has low affinity for IL-6R when compared with hIL-6 (Friborg et al., 1999).

vIL-6 induces angiogenesis by VEGF expression using similar mechanisms as hIL-6 and can therefore cause further increase in expression of hIL-6, suggesting that ‘molecular mimicry’ is also an important factor in the pathogenesis and evolution of MCD (Aoki et al., 1999).
It also stimulates the JAK/STAT pathway via STAT3 signalling which results in increased cell growth (Aoki et al., 2003). The rate of expression of vIL-6 is much higher in MCD than in KS (only 1–2% of cells express vIL-6) or primary effusion lymphoma (Cronin & Warnke, 2009). Various studies, using quantitative polymerase chain reaction in peripheral blood mononuclear cells and plasma, have shown that there is an association between high copy numbers of HHV-8 and increased plasma IL-6 levels, both of which correlate with disease exacerbations and symptom flares (Grandadam et al., 1997; Oksenhendler et al., 2000). vIL-6, which is also believed to be a key player responsible for B-cell proliferation, is also frequently expressed on LANA-1 positive cells and its expression has been reported to confer a worse prognosis (Parravicini et al., 1997).

Parravicinni et al. (1997) further demonstrated, using specific double immunostaining in CD, that some lymphovascular endothelial cells showed co-expression of LANA-1 and vIL-6. It is can also be expressed by a significant proportion of latently infected cells in MCD. In lymphoid cells, vIL-6 is expressed mainly around the follicles and within the follicular dendritic network (Talat & Schulte, 2011). It was also shown that vIL-6 was likely to contribute to neoplastic lymphoproliferations (Parravicini et al., 1997). Both hIL-6 and vIL-6 are sufficient to induce disease flares in HHV-8 associated MCD with the increased expression of other proinflammatory cytokines (Reddy & Mitsuyasu, 2011).

1.6.3.3 **IL-1 and NFκB**

There are two distinct IL-1Rs: IL-1RI and IL-1RII (Gherardi et al., 1994). IL-1 usually binds to IL-1RI, triggering a signalling cascade in the cytoplasm leading to the activation of I-NFκB kinase (IKK). NFκB is released and enters the nucleus to turn on several genes that regulate cell functions, especially those encoding IL-1 and IL-6 (El-Osta et al., 2010). It is likely that IL-1 and IL-6 are both overexpressed in CD because of aberrant upstream regulation or due to upregulation of IL-1 that eventually leads to elaboration of IL-6 (El-Osta & Kurzrock, 2011).

1.6.4 **Growth factors**

1.6.4.1 **VEGF**

As previously described, especially with reference to the HV-CD, blood vessel proliferation is an important histologic component although the role of angiogenesis in the pathophysiology of CD has received less attention than IL-6. Nishi & Maruyama (2000)
demonstrated that increased VEGF expression is present in the interfollicular areas of lymph nodes in patients with CD, with some patients even having elevated systemic VEGF levels. VEGF was found to be strongly expressed in plasma cells in CD but rarely expressed in normal lymph nodes (Nishimoto et al., 2000). Dysregulated IL-6 gene expression is considered to be a primary event that is related to paracrine VEGF production by plasma cells and variable vascular proliferation in lymph nodes (Chen et al., 2009). This was reinforced by Nishi & Maruyama (2000) who examined one patient with the unicentric PC-CD and one patient with MCD, both who had elevated levels of serum VEGF which then fell to normal levels after nodal resection and chemotherapy respectively.

1.6.4.2 Epithelial growth factor receptor (EGFR)
EGFR has been a therapeutic target in the treatment of various cancers. Sun et al. (2003) evaluated EGFR expression in follicular dendritic cells and surrounding perifollicular reticular cells in patients with CD and found EGFR expression in all 24 cases. The significance of immunohistochemical overexpression of EGFR in relationship to therapeutic targeting is less clear than that for EGFR mutations (El-Osta & Kurzrock, 2011).

1.6.5 B-cell populations
CD20 is a phosphoprotein expressed on the surface of mature B lymphocytes. Immature B-cells are abundant in the germinal centres of lymph nodes where they interact with antigen presenting follicular dendritic cells and follicular T-helper cells (Beltman et al., 2011). Dupin et al. (2000) demonstrated that lymph nodes from patients with HHV-8 associated MCD harbour HHV-8 in plasmablasts localised in the mantle zone. These plasmablasts were IgM restricted and also showed variable expression of CD20. HHV-8 infected plasmablasts can express CD20 as well as B-lymphocyte-induced maturation protein 1 (BLIMP1) (Naresh et al., 2009; Shulte and Talat, 2010). Recent data by Knowlten et al. (2014) shows that HHV-8 infected B-cells display a polyfunctional cytokine profile within a background of increased IL-6, tumour necrosis factor (TNF), macrophage inhibitory protein-1 (MIP-1) and IL-8 which occurs after HHV-8 infection as well as in the blood of HIV/HHV-8 co-infected patients with KS. Campbell et al. (2014) postulated that activated B-cells within a chemokine rich environment is an important trigger that drives endothelial cell proliferation in KS.
This mechanism could be responsible for the development of HHV-8 associated malignancies. B-cells could also serve as a reservoir of latently infected HHV-8 cells as it has been reported that LANA-1 was detected in IgM+ B-cells of tonsil origin (Hassman et al., 2011). B-cells, localised to the mantle zone of B-cell follicles, are also known to harbour the HHV-8 virus in individuals with KS, PEL, and MCD (Ambroziak et al., 1995; Cesarman & Knowles, 1999). A study conducted by Chadburn et al. (2008) on 17 cases of HIV-positive MCD indicated that although HIV-positive MCD originated in pre-terminally differentiated B-cells, it may in fact arise from extrafollicular B-cells. These cells were characterised by OCT2, cytoplasmic lambda immunoglobulin expression, variable B-cell marker expression, lack of CD138 and EBV negativity.

Rituximab, an anti-CD20 antibody, is currently used in CD to induce lymphodepletion and subsequent decrease of IL-6 production, not only in HHV-8 positive patients but also in HHV-8 negative patients (El-Osta & Kurzrock, 2011). This is supported by numerous case series and two open-labelled studies that have successfully used Rituximab (Bower, 2010). One study of 21 patients with newly diagnosed HIV-associated MCD reported a radiological response rate of 67% with the overall and disease-free survival rates at 2 years being 95% and 79%, respectively (Bower et al., 2007). The second prospective study, which enrolled 24 HIV-positive MCD patients classified as chemotherapy dependent, showed that Rituximab induced a sustained remission of 1-year duration in 17 of 24 (71%) patients with a 92% 1-year overall survival (Gerard et al., 2007). These findings support the dual role played by B lymphocytes in both disease pathogenesis and in providing a reservoir for HHV-8 activation.
1.7 DC-SIGN

Dendritic cell-specific intercellular adhesion molecule (ICAM)-grabbing non-integrin (DC-SIGN) is a mannose-specific C-type lectin expressed by dendritic cells. C-type lectins are calcium-dependent carbohydrate-binding proteins with a wide range of biological functions, many of which are related to immunity (Soilleux, 2003). DC-SIGN (CD209) is expressed on myeloid dendritic cells (DCs) in the dermis, mucosa, lymph nodes, lung and thymic T-lymphocytes (Soilleux, 2003).

1.7.1 Functions of DC-SIGN

The diverse and specialised functions of DCs and its widespread distribution in the body play important roles in bridging of the two arms of the immune system to obtain a defence against foreign antigens. The various types of DCs, namely myeloid dendritic cells (mDC), Langerhans cells (LC), skin dermal dendritic cells (DDC) and submucosal dendritic cells (SMDC) all have essential roles in both innate and adaptive immune
responses to primary and subsequent infections as well as the reactivation of chronic viral infections (Campbell et al., 2014). They act during the innate immune response as specialised cells that survey and detect antigens of foreign microorganisms throughout the body which induces their ability to communicate with helper and effector lymphocytes thus bridging the innate and adaptive response (Campbell et al., 2014).

In the course of dendritic cell life cycle, DC-SIGN has emerged as a prominent receptor on DCs that mediates many critical interactions, including antigen uptake and presentation and transendothelial migration to lymph nodes (Su et al., 2003). DC-SIGN also binds to ICAM-3 on T-lymphocytes, thereby playing an important role in the activation of T-lymphocytes (Soilleux, 2003). DC-SIGN and other C-type lectins act as pathogen recognition receptors that alert macrophages and DCs to take up and process pathogens for antigen presentation to T-cells (Geijtenbeek et al., 2002).

![Figure 2: Structure of DC-SIGN. DC-SIGN, composed out of four domains: (A) cytoplasmic domain, (B) transmembrane domain, (C) 7 or 8 extracellular neck repeats and (D) carbohydrate recognition domain (Alen and Schols, 2012)](image)

### 1.7.2 DC-SIGN and HIV

DC-SIGN also plays a pivotal role in HIV-1 infection as a DC-specific HIV-1 binding protein. It also enhances HIV-1 infection of T-cells (Geijtenbeek, et al., 2000; Lin et al., 2003). The tissue distribution of immature DCs maximizes its chance to be bound by incoming virions being deposited in mucosal surfaces (Su et al., 2003). It is present at sites of first exposure to HIV, including mucosal tissues of the cervix, rectum and uterus. It is also expressed on macrophages of the lung alveoli, placenta, inflammatory lesions, and IL-13 activated, monocyte-derived macrophages (Soilleux, 2003). The glycoprotein gp120 selectively binds to the carbohydrate recognition domain (CRD) of DC-SIGN with
high affinity and is also known to bind HIV for protracted periods (Su et al., 2003; Rappocciolo et al., 2008).

HIV has exploited DC-SIGN to perpetuate its own pathogenic life cycle by exploiting DC migration through the body. It does this by binding DC-SIGN on the surface of immature DC via gp120 and enhancing its maturation which allows the virus to target the CD4+ T-cells (Soilleux, 2003). Two types of DC-mediated HIV transmission have been proposed: trans-infection and cis-infection (Rappocciolo., 2006). Trans-infection mediated by DCs can occur by two pathways: i) HIV transmission occurs across the infectious synapse whereby DCs transfer captured HIV to target CD4+ T-cells through cell-cell junctions known as infectious synapses which are formed by DC-SIGN; ii) HIV transmission occurs by an exocytic pathway that involves HIV-associated exosomes. Exosome-associated HIV virions are likely to be transmitted to CD4+ T-cells through membrane binding and fusion. Cis-infection mediated by DCs occurs when there is HIV infection of DCs after initial exposure with replication in DCs resulting in de novo production and long-term transmission of HIV (Karn and Stoltzfus., 2012).

HIV most likely has evolved strategies to specifically exploit the use of the DC-SIGN in its trans-infection process. This exploitation specifically involves the Nef viral gene whereby expression of this gene in DCs increases the number of DC-SIGN molecules on the cell surface (Su et al., 2003). This enhanced cell surface expression of DC-SIGN is advantageous to the virus since this increases the binding affinity to T-cells and therefore increases the chance the virus has to find its receptors on T-cells.

### 1.7.3 DC-SIGN and HHV-8

Several receptors in the extracellular matrix have been implicated in HHV-8 entry into different human cell types. It has been demonstrated that DC-SIGN is a cellular receptor for HHV-8 (Rappocciolo et al., 2006a; Rappocciolo et al., 2008; Knowlton et al., 2014). In addition to its expression on monocyte-derived dendritic cells (MDDCs), DC-SIGN is also expressed on activated macrophages and B-cells (Soilleux et al., 2000; Rappocciolo et al., 2006; Rappocciolo et al., 2008). These cell types represent natural targets for HHV-8 in vivo. Studies on the interactions between DC-SIGN and other viruses known to use DC-SIGN as an entry receptor, such as HIV, Ebola, hepatitis C and cytomegalovirus (CMV), have demonstrated that viral glycoproteins are the viral attachment proteins
responsible for binding to DC-SIGN, or its endothelial cell-expressed homologue, DC-SIGNR (Geijtenbeek et al., 2000; Pohlmann et al., 2000; Lin et al., 2003). The majority of these studies have demonstrated that viral glycoproteins with a high mannose glycan structure, like HHV-8, bind to DC-SIGN/DC-SIGNR (Anderluh et al., 2012).

Establishment of HHV-8 infection requires two separate events at the surface of susceptible cells which includes binding to an attachment receptor which is followed by binding to entry receptors (Campbell et al., 2014). Although Langerhans cells do not express the HHV-8 entry receptor DC-SIGN, they do express the HHV-8 attachment receptor HS on their surface, which the virus uses before entry into the cell (Soilleux & Coleman, 2001; Hengge et al., 2002b). Initially, the host immune system is able to control the infection, however following an immune compromising event, such as organ transplantation or HIV infection there is reduced cellular antiviral response against HHV-8 which is worsened by abnormal cytokine elevations (Campbell et al., 2014). Reports support that HHV-8 is capable of recognising multiple receptors on human host cells and that HHV-8 employs receptor flexibility to expand the range of cell types it infects while at the same time adapting to different immunological environments that it encounters in the host (Hengge et al., 2002a).

Studies done by Angel et al. (2009) demonstrated that DC-SIGN expressing antigen presenting cells (APCs) were located in the medullary cords and lined the capsule and trabeculae of lymph nodes. These DC-SIGN+ APCs were also consistently found scattered throughout the paracortex of all lymph nodes examined, where they tended to wrap around high endothelial venules. The subcapsular APCs also extended along trabeculae radiating into the lymph node as well as the capsule. APCs expressing a low level of DC-SIGN were scattered around the medullary cords and in the perifollicular zone of the paracortex. Plasmacytoid DCs (pDC) are a lymphoid-lineage subset of APCs that produce extraordinary amounts of the antiviral protein, interferon α (IFN-α) in response to virus infection (Liu et al., 2013). Although pDC do not express DC-SIGN, it is known that pDC precursors express DC-SIGN, indicating they could also be susceptible to HHV-8 infection (Campbell et al., 2014).
1.7.4 DC-SIGNR

A homologue of DC-SIGN, termed DC-SIGNR (for DC-SIGN related) (CD209L) or L-SIGN (for liver/lymph node-specific ICAM-3 grabbing non-integrin) exhibits and shares 77% amino acid identity with DC-SIGN with the greatest areas of homology located within the C-type lectin domain and the neck region (Geijtenbeek et al., 2002). DC-SIGNR is expressed on endothelial cells in liver sinusoids, lymph node, gastrointestinal tract, and placenta (Soilleux, 2003). Endothelial cells have been speculated to play a similar role to macrophages and DCs in antigen capture and clearance from the blood. DC-SIGNR, like DC-SIGN, binds preferentially to high-mannose oligosaccharides through a carbohydrate recognition domain in the C terminus (Pohlmann et al., 2000). Studies have found that DC-SIGNR functions similarly to DC-SIGN with regards to virus attachment and transmission, as might be expected given the high degree of homology between these molecules (Soilleux et al., 2000).

1.8 HIV/HHV-8-associated MCD

The diagnosis is often easily made in the appropriate high risk setting and is usually consistent with an inflammatory process characterised by fevers, multifocal lymphadenopathy, hepatosplenomegaly, oedema, severe cytopaenia, high serum C-reactive protein levels, elevated HHV-8 DNA levels in PBMCs, extreme plasmacytosis in lymph nodes or bone marrow, Kaposi sarcoma and haemophagocytic syndrome (Mylona et al., 2008). Research has shown that the exacerbation of clinical symptoms in HIV infected patients with MCD was associated with large increases in the HHV-8 viral loads in their PBMCs (Grandadam et al., 1997; Oksenhendler et al., 2000; Collins et al., 2006). Plasma levels of HHV-8 DNA predict relapse rates in HIV-associated MCD (Stebbing et al., 2011).

Patients with HIV-associated MCD are at an increased risk of developing non-Hodgkin lymphoma (NHL). A prospective cohort study of 60 HIV-positive MCD patients found that these patients have a 15-fold increased risk of developing HHV-8 associated non-Hodgkin lymphoma as compared with the general HIV-positive population (Oksenhendler et al, 2002). Three developed primary effusion lymphoma, five developed extranodal high grade B-cell lymphomas and six were diagnosed with an aggressive plasmablastic lymphoma. It was estimated from this series that the 2-year probability of developing NHL after MCD diagnosis was 24% and neither plasma HIV RNA level nor CD4 count
was predictive. There is also an observable increase in occurrence of KS in HIV-associated CD patients and KS foci have been described in the lymph nodes of nearly two thirds of patients (Lachant et al., 1985; Oksenhendler et al., 1996; Naresh et al., 2008; Mylona et al., 2008).

Large population studies have also revealed an increased incidence of HIV-associated CD since the introduction of HAART, which appears to have no effect on the progression of the disease (Stebbing et al., 2008; Pinto and Nunes, 2011). However, a study of 84 CD patients by Mylona et al. (2008) showed that life expectancy in MCD appeared to have improved, with a mortality rate of 29% among patients receiving ART compared to a mortality rate of 75% among pre-ART patients. Another significant finding in their study was that patients on ART treatment at the time of diagnosis of MCD had a better immunological profile and were less likely to have concurrent KS than those commencing ART after the diagnosis of MCD was made (Mylona et al., 2008). The introduction of HAART has been associated with a reduction in the incidence of many HIV-associated malignancies including KS as it causes a decrease in HHV-8 viral replication and prolonged time to progression (Bower et al., 1999).

1.9 Associated conditions

There are a variety of related processes and other disease associations, some of which stem from the immune dysregulation that occurs while other conditions may be direct manifestations of the CD.

1.9.1 Kaposi sarcoma

Kaposi sarcoma was first described by Moritz Kaposi in 1872 as "idiopathic multiple pigmented sarcomas of the skin" and was characterised by angioproliferative, tumour-like lesions usually developing first in the skin with eventual dissemination to multiple cutaneous sites, viscera and lymph nodes (Kaposi, 1872). Although previously a relatively rare disease, it is now a global health problem because of its association with the HIV pandemic and other immunosuppressed states.
There are four clinically recognised KS types:

i) Classical or sporadic KS was initially described as a slow growing, indolent tumour mostly developing in the extremities of elderly males of eastern and Mediterranean Europe (Kaposi, 1872)

ii) Endemic KS is clinically more aggressive and predominantly seen in eastern and central sub-Saharan Africa before the AIDS epidemic. This type is clinically similar to classical KS, but can also present with a more fulminant and fatal form in children (Dedicoat & Newton, 2003). The childhood endemic KS subtype often involves lymph nodes with or without skin involvement.

iii) Iatrogenic KS is often seen as a consequence of drug related immunosuppression following transplantation, and normally regresses upon immune reconstitution (Mendez & Paya, 2000).

iv) AIDS-associated KS is the most frequent tumour diagnosed in the setting of HIV infection and the most aggressive form of KS with early dissemination in the skin and viscera (Levine, 1992; Pyakurel et al., 2006). This type frequently resolves upon treatment of HIV by antiretroviral combination therapy and the resulting immune reconstitution.

Although these four clinical forms differ with regard to geographic distribution, aggressiveness and localisation, all display common clinical features and are histologically indistinguishable (Gramolelli & Schulz, 2015).

In the setting of MCD, recurrent cutaneous and nodal forms of KS have been reported, being present at diagnosis or subsequently developing in 75% of patients with HIV-associated MCD and 13% of HIV-negative patients with MCD (Soulier et al., 1995a/b; Oksenhendler et al., 1996). Circulating HHV-8 infected cells are hypothesised to enter the lymph node through afferent lymphatics with early KS usually localising within the subcapsular and trabecular sinuses while advanced KS is observed to efface the majority of the node (O’Leary et al., 2000). There is infection of both nodal smooth muscle and endothelial cells which eventually become spindled in morphology (Brousset et al., 2001). With progressive infection, small foci of spindle cells eventually appear in the subcapsular space of the node and interestingly reflect the highest copy number of HHV-8 (O’Leary et al., 2000). KS, within the context of HIV-associated MCD, is often characterised by microscopic involvement of lymph nodes and spleen.
Although the co-existence of these two diseases within the same tissue has been observed, there are few actual reports in the literature with only a few case series immunohistochemically evaluating the presence of the two lesions within the same tissue specimen (Abe et al., 2006; Naresh et al., 2008; Pinto & Nunes, 2011). Naresh et al. (2008) investigated the co-existence of the two entities in 24 lymph nodes and five spleens in patients with HIV-associated MCD.

Among the lymph node specimens, 63% had additional microscopic involvement by KS involving the capsule, trabeculae and hilar regions. This group was compared to 20 lymph nodes of HIV positive patients without evidence of CD. Only 25% of the second group showed microscopic involvement by KS, which was significantly lower than in the group with MCD. Another study by Abe et al. (2006) reported a case of KS and MCD in a single lymph node from a HIV positive patient, where immunohistochemistry was used to demonstrate the differential expression of the viral proteins in the two lesions. It was found that MCD showed expression of both lytic and latency phase genes of HHV-8 within the cells of the mantle zone, while the neoplastic cells within the KS lesions were predominantly positive for latency protein LANA-1 (Abe et al., 2006).

In another similar study by O’Leary et al. (2000) researchers looked at 16 patients with CD and examined the correlation between HHV-8 and lymph node angiogenesis. Of the study sample, 43% showed positive HHV-8 staining with further detailed analysis confirming that the virus was identified in 10% of the B-lymphocytes. Other studies, like the one conducted by Brousset et al. (2001), used double staining to evaluate the expression LANA-1 and vIL-6 in cases of MCD and KS. They observed that approximately one-third of the lymphoid cells were positive for both markers in MCD, confirming both lytic and latent phase gene expression within these cells.

1.9.2 Non-Hodgkin lymphoma

Patients with MCD often develop secondary tumours, such as KS, NHL, Hodgkin lymphoma and plasmacytoma (Oksenhendler et al., 1996). Talat and Schulte (2011) reported that NHL has been reported in <5% of HIV-negative CD compared to 20% in HIV-associated CD. Oksenhendler et al. (2002) found that the HIV viral load or CD4 count was not predictive of the risk of developing NHL and their prospective study of 60 patients with HIV-associated MCD found that 23% developed NHL while up to 50%
developed aggressive plasmablastic lymphoma over a median period of 20 months. In addition, there are lymphomas that arise de novo in the setting of MCD, that are now recognized in the current World Health Organization classification as large B-cell lymphoma arising in HHV-8 associated MCD (Swerdlow et al., 2008).

1.9.3 POEMS and TAFRO

MCD is often associated with autoimmune signs and symptoms and MCD patients often have some manifestations of POEMS syndrome (Dispenzieri, 2011; Muskardin et al., 2012). POEMS syndrome is a rare, devastating, multisystemic disease that occurs in the setting of a plasma cell dyscrasia.

A few large case series have documented MCD in 11–24% of POEMS cases (Soubrier et al., 1994; Dispenzieri et al., 2003). Enlargement of the lymph nodes and spleen in most patients with POEMS syndrome is mainly secondary to plasma cell type MCD. In a 30-year retrospective study, 37% of MCD patients had concurrent POEMS syndrome (Shin et al., 2011). However, peripheral neuropathy and monoclonal paraprotein with or without other features of POEMS are only rarely seen in patients with HIV (Shin et al., 2011).

IL-6 may cause immune dysregulation, leading to autoimmune phenomena, such as, autoimmune haemolytic anaemia, thrombocytopenia, pure red cell aplasia, acquired factor VIII deficiency, systemic lupus erythematosus and myasthenia gravis (Reddy & Mitsuyasu, 2011).

TAFRO syndrome is a recently described condition characterised by concurrent generalised lymphadenopathy showing MCD features, and a constellation of symptoms that include thrombocytopenia, anasarca, fever, reticulin fibrosis and organomegaly (TAFRO). TAFRO syndrome usually occurs in the middle-aged and elderly with a median age of 56 years. It has a female predilection (4:1) and is not associated with HHV-8, HIV or EBV (Kawabata et al., 2013). The distinct clinicopathological features suggest that the TAFRO syndrome may represent a distinct subtype of what is currently considered iMCD.
1.10 Treatment modalities

1.10.1 Unicentric disease
The preferred management of UCD is complete surgical excision, which is curative in approximately 95% of patients and affords resolution of constitutional symptoms, if present (Keller et al., 1972). Active surveillance and long-term follow-up is recommended because recurrences that occur as late as 11 years after incomplete resection have been reported (Chronowski et al., 2001). UCD also responds to local ablation therapy in cases where patient or anatomical factors make surgical resection difficult (Talat & Schulte, 2011).

1.10.2 Multicentric disease
Until recently, MCD was not considered an AIDS-related or AIDS-defining event, and was often managed with local surgery, irradiation and corticosteroids. Cytotoxic therapy was reserved only for highly symptomatic patients with refractory disease. Historically, the prognosis has been poor, with a median survival not exceeding 25 months (Oksenhendler et al., 1996; Oksenhendler, 2009). However, the prognosis has improved recently with the use of HAART and advanced therapeutic algorithms (Van Rhee et al., 2010).

As MCD is almost always symptomatic, therapy is recommended. A wide variety of treatment options are available, which includes supportive care. Therapy is aimed at eradicating the underlying viral infections, modifying IL-6 cytokine dysregulation, reduction of proliferating B-cell populations, debulking of tumour mass and improving immunocompetency. Options for therapy include antiviral and antiretroviral agents, rituximab, chemotherapy, monoclonal antibodies, immune modulators and corticosteroids (Bower, 2010). Chemotherapy has evolved from single-agent (e.g. chlorambucil) to combination chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisone – CHOP), to the current use of rituximab and etoposide (Gerard et al., 2007; Bower, 2010). The literature however shows that although there are some strong responses, these responses are incomplete or short lived (Kaplan, 2013).

1.10.2.1 HIV-positive/HHV-8 positive MCD
The use of HAART to treat patients with HIV/AIDS has altered the natural history of HIV and has increased overall survival and reduced the progression rate to lymphoma, which is
10-20 times lower since the introduction of HAART (Oksenhendler, 2009). Unlike early series in which median survival of MCD patients was about poor, overall survival with current targeted therapies exceeds 85% at 1 year and sustained responses are often seen (Uldrick et al., 2012). Active lytic HHV-8 replication is highest in MCD and disease flares are usually associated with an increased HHV-8 viraemia.

As the pathogenesis of MCD also reflects HHV-8 infection of B-cells in affected lymph nodes, the use of anti-CD20 monoclonal antibodies to eliminate these pathogenic cells can be used as a treatment option. Rituximab therapy, either alone or in combination with chemotherapy (R-CHOP), appears to be a promising first line treatment for HIV-associated MCD (Bower, 2010). The role of Rituximab in the treatment of HIV-associated MCD is supported by prospective and retrospective trials demonstrating sustained remissions (Powles et al., 2009; Bower et al., 2011). It should be noted that patients with MCD and concurrent KS may experience significant progression of KS after rituximab therapy (Kaplan, 2013).

The promising clinical efficacy exhibited by targeting IL-6 or IL-6R has subsequently shown that IL-6 is an important target in the treatment of CD and monoclonal antibodies (anti-IL-6 & anti-IL-6R), such as Siltuximab, have proven to be successful targeted therapy options. The correlation between the degree of proliferation of HHV-8 and symptom severity and symptom regression after administration of antiviral agents provides evidence of the critical role of HHV-8 in CD.

1.10.2.2 *HIV-negative /HHV-8-negative MCD*

Three main treatment strategies have been employed based on the current understanding of iMCD. These include the specific use of anti-inflammatory and immunomodulatory therapies to assist in the elimination of cells responsible for hypercytokinaemia as well as monoclonal antibodies, such as Tocilizumab and Siltuximab, for the blockade of aberrant IL-6 signalling (Fajgenbaum et al., 2014). Although both these mAbs have shown clinical activity in iMCD and are potential options for frontline therapy, they do require life-long administration and may not be effective in all patients. For those patients that are anti-IL-6 refractory, therapeutic approaches that target pathways upstream of IL-6 are being explored.
The US Food and Drug Administration (FDA) has approved siltuximab for the treatment of patients with HIV negative, HHV-8 negative MCD, where it shows significant clinical activity, resulting in control of IL-6 dependent systemic symptoms and laboratory abnormalities (Kurzrock et al., 2013). Although there was improvement in symptoms, such as, fever, fatigue, anaemia, lymphadenopathy and hypergammaglobulinaemia, remissions were not sustained and recurrences were observed (Kaplan, 2013).

Conventional anti-inflammatory and immunosuppressive therapies are currently being used more frequently as some physicians are treating iMCD more like a systemic inflammatory disease (Liu et al., 2016). Rituximab, which is a frequent first or second-line therapy in iMCD, is only partially effective and typically does not provide long-term disease control.

Cytotoxic lymphoma-based chemotherapies, such as CHOP can be effective in a proportion of severely ill iMCD patients by reducing a large number of cytokine-secreting cells, although relapses are common and side effects are significant. Novel therapies targeting other cytokines and intracellular signalling pathways are also being investigated as possible therapeutic options.

It is of relevance that iMCD treatment can cause or even increase susceptibility to secondary malignancies with haematological malignancies being diagnosed in an increased frequency within 2-years of iMCD diagnosis (Fajgenbaum et al., 2014).

1.11 Study justification

The exact prevalence and incidence of CD in South Africa and sub-Saharan Africa has not yet been established. The number of HIV/HHV-8 MCD cases diagnosed at GSH is increasing and it is my opinion that the high prevalence of HHV-8 in the Western Cape is directly associated with HIV-related CD. Although it occurs more frequently in HIV-infected individuals than those without HIV infection, the poor prognosis and often fatal course, is not fully explained by the underlying HIV alone. There are still many unanswered questions related to viral biology, aetiopathogenic kinetics and cellular targets implicated in CD. One of the main cellular targets common to both viruses is DC-SIGN and its related DC-SIGNR. Our hypothesis is that both HIV and HHV-8 s manipulate DC-
SIGN and DC-SIGNR to gain access into susceptible cells, which allows for both immune evasion and the development of abnormal lymphoproliferations observed in CD.

1.12 Aims and Objectives
To determine the role that DC-SIGN and DC-SIGNR play in HHV-8 infection of target cells by demonstrating their presence in LANA-1 positive cells. We also wanted to determine whether HHV-8 and HIV co-infection occurs in the same cells by identifying dual LANA-1 and p24 expressing cells in addition to verifying if it was B- and/or T-lymphocytes that infected by HHV-8 by using CD20 and CD3.
CHAPTER 2: 
MATERIALS AND METHODS

2.1 Ethics approval
Ethics approval was obtained from the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (Reference number 144/2012; see Appendix 2). Scientific approval was obtained from the Department of Pathology Research Committee.
The study was funded by the National Health Laboratory Service Research Trust.

2.2 Study design
This was a retrospective descriptive and analytic cross-sectional immunohistochemistry study.

2.3 Sample selection
An electronic SNOMED search of the DISA laboratory information system (LIS) database of the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur Hospital (GSH) was performed to identify all cases of Castleman disease from 2003 to 2013. Inclusion criteria included all CD cases diagnosed during study period. Exclusion criteria included needle core biopsy specimens. Archived stained slides of the cases were retrieved and reviewed by the study investigators. The diagnosis in each case was reviewed. Other relevant information that was recorded onto Excel data spreadsheet (Appendix 1) included:
• Age and sex of patient
• HIV status (positive, negative or unknown)
• CD4 count
• Concurrent HAART
• Castleman disease subtype
• Topographic lymph node region excised with specific descriptors (localised vs generalised lymphadenopathy)
• Previous or concomitant diagnosis of Kaposi sarcoma or non-Hodgkin lymphoma
Data for most of the variables except for CD4 count and HIV status were obtained from the histology reports. The HIV status and CD4 counts, where available, were obtained from relevant serological investigations and CD4 reports available on the DISA LIS.

During review of the haematoxylin and eosin (H&E) sections a suitable block of tumour tissue was selected for immunohistochemical staining. Formalin fixed paraffin wax embedded tissue (FFPE) blocks were then retrieved from the archives of the Division of Anatomical Pathology and 3μm sections were cut for immunohistochemistry.

Cases were allocated study numbers and patients’ names and other identification details were removed.

2.3.1 Reliability of data
Demographic data entered onto the DISA LIS were obtained from the requisition forms completed by the submitting clinical doctor at the time of lymph node excision. The reliability of data for this study is therefore dependent on the accurate submission of information supplied especially with regard to variables such as age, sex and biopsy topographic sites. The accuracy of data obtained from the DISA system is also reliant on data capturers who entered details from requisition forms.

2.4 Morphological analysis
To avoid bias, the morphological review was conducted without knowledge of the original diagnosis. Cases showing atrophic hyalinised germinal centres, hypervascularisation and FDC prominence were classified as HV-CD. Cases exhibiting hyperplastic germinal centres, profuse plasmacytosis that expanded the interfollicular zones and a population of larger cells, plasmablasts (plasmacytoid immunoblasts), that localized to the mantle zone or paracortical regions were classified as PC-CD. These plasmablasts are at least twice the size of reactive mantle zone lymphocytes and are characterised by a moderate amount of amphophilic cytoplasm, large vesicular nucleus and one or two prominent nucleoli. PC-CD cases showing larger aggregates of these ‘plasmablasts’ both within the mantle and interfollicular zones and expressing LANA-1 by immunohistochemistry were then further subclassified as plasmablastic CD (Pb-CD). For purposes of this study, cases that showed
a combination of both HV-CD and PC-CD features were classified as mixed CD. All cases were assessed for the presence of concurrent KS or NHL.

2.5 Immunohistochemistry

2.5.1 Antibodies

Table 1: Primary antibodies

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>CLONE</th>
<th>CLONALITY</th>
<th>DILUTION</th>
<th>INCUBATION</th>
<th>SUPPLIER</th>
<th>CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-8-LANA (M)</td>
<td>13B10</td>
<td>Monoclonal</td>
<td>1:50</td>
<td>1 Hr at RT</td>
<td>Leica, Newcastle, UK</td>
<td>Lymph node</td>
</tr>
<tr>
<td>DC-SIGN CD209 (M)</td>
<td>5D7</td>
<td>Monoclonal</td>
<td>1:10</td>
<td>Overnight at RT</td>
<td>Abcam, Boston, USA</td>
<td>Lymph node</td>
</tr>
<tr>
<td>DC-SIGNR CD299 (M)</td>
<td>EPR11211</td>
<td>Monoclonal</td>
<td>1:100</td>
<td>Overnight at RT</td>
<td>Abcam, Boston, USA</td>
<td>Lymph node</td>
</tr>
<tr>
<td>HIV1-p24 (M)</td>
<td>05-001</td>
<td>Monoclonal</td>
<td>1:50</td>
<td>Overnight at RT</td>
<td>Santa Cruz, Spain, Europe</td>
<td>Lymph node</td>
</tr>
</tbody>
</table>

(Key: M – mouse; RT – room temperature)

Four monoclonal antibodies (mAbs) were used in this study (Table 1).
Anti-LANA-1 is the mAb directed against the latent nuclear antigen-1 (LANA-1) which is encoded by HHV-8 ORF 73.

Anti-p24 is the mAb directed against the extracellular domain of the HIV-1 viral core protein. Anti-DC-SIGN (CD209) and anti-DC-SIGN related (CD299/CD209L) are mAbs targeting human cellular receptors on dendritic cells.

2.5.2 Controls

A negative reagent control, in which the primary antibody was replaced by phosphate buffered saline solution (PBS) and an appropriate positive tissue control were run simultaneously for each batch of staining.
2.6 Immunohistochemical methods

2.6.1 Immunohistochemistry – Single antibody method

Table 2: Kits used for the IHC protocol

<table>
<thead>
<tr>
<th>KITS</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Envision HRP System Labelled Polymer Anti-mouse</td>
<td>Dako - CA, USA</td>
</tr>
<tr>
<td>Envision G/2 Double Stain System (Rabbit/Mouse)</td>
<td>Dako - CA, USA</td>
</tr>
<tr>
<td>Liquid DAB + Substrate chromogen system</td>
<td>Dako – CA, USA</td>
</tr>
</tbody>
</table>

- Three micron tissue sections were cut from FFPE blocks, picked up onto Histobond slides (Marienfeld-Germany) and heat fixed on a hotplate at 60°C for 10-15 minutes.
- Sections were dewaxed through xylene, cleared in ethanol and rehydrated in water.
- Endogenous peroxidase activity was blocked by treating the slides with a 3% hydrogen peroxide (H$_2$O$_2$) solution for 10 minutes.
- Slides were washed well in water.
- Antigen retrieval was performed by pressure-cooking slides in Tris-EDTA (pH9) for 1 minute 30 seconds at full pressure.
- The sections were allowed to cool for 10 minutes.
- This was followed by washing in tap water.
- Thereafter, slides were rinsed with PBS (pH 7.6) (Oxoid-Hampshire, England).
- Non-specific binding was blocked by treating slides with a 5% goat serum solution (DAKO - Denmark).
- Serum was then drained off and sections were incubated with primary antibody at room temperature at specified times and dilutions (Table 1).
- The slides were then washed well with PBS.
- This was followed by incubation with DAKO Envision labelled Polymer, HRP (DAKO- USA) for 30 minutes at room temperature.
- Sections were washed well with PBS.
- Positivity was developed by applying the chromogenic substrate 3,3 diaminobenzidine (DAB) (DAKO - USA) for 10 minutes.
- Slides were washed in running tap water and counterstained with Mayers haematoxylin for approximately 3 minutes.
• After washing in running tap water, sections were blued in ammoniated water.
• Finally, the slides were then dehydrated through alcohols, cleared with xylene and mounted with Entellan (MERCK- Germany).

2.6.2 **Envision G/2 DoubleStain System (DAKO) Method (Table 2)**
• FFPE tissue sections were cut at 3µm and picked up onto Histobond slides (Marienfeld-Germany).
• The slides were then dewaxed through xylene and cleared in alcohol, with a final thorough wash in running tap water.
• Antigen retrieval was performed in a pressure cooker for 1 minute 30 seconds using Tris EDTA pH9 as the retrieval buffer.
• A 3% H₂O₂ solution was used to remove endogenous peroxidase for 10 mins.
• After washing in water the slides were rinsed in DAKO wash buffer (DAKO, Germany) for 5-10 mins.
• This was followed by incubation in 5% goat serum for 10-15 mins.
• The appropriate first primary antibody (Table 3) was then applied to each slide according to the dilutions and time specified (Table 1).
• After rinsing slides in DAKO wash buffer, Polymer HRP was then applied and left for 10-20 mins.
• DAB was used as the chromogen and was left to develop for 10 mins.
• A wash in DAKO wash buffer solution followed for 5-10 mins.
• The doublestain block was put on slides for 3mins.
• This was followed by incubation with the second primary antibody (Table 3) according to the recommended conditions (Table 1).
• Slides were then rinsed with DAKO wash buffer.
• Rabbit/Mouse (LINK) solution was added and incubated for 10-20 mins (Table 2)
• The polymer alkaline phosphatase (AP solution) was applied to slides for 10-15 mins.
• Permanent Red was used as the second chromogen (Table 3) and incubated for 10 mins.
• Slides were counterstained in haematoxylin for 3 mins
• Finally, the slides were coverslipped using glycergel (DAKO, Germany) as the mounting medium.
2.7 Dual immunohistochemical staining profiles

The dual immunoreactivity for each antibody pair, as listed below, was assessed.

- DC-SIGN and LANA-1
- DC-SIGNR and LANA-1
- p24 and LANA-1
- CD20 and LANA-1
- CD3 and LANA-1

Table 3: Localisation of double antibody staining

<table>
<thead>
<tr>
<th>Combination of Antibodies</th>
<th>First primary antibody</th>
<th>Second primary antibody</th>
<th>Staining profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>LANA-1 and DC-SIGN</td>
<td>LANA-1 (DAB)</td>
<td>DC-SIGN (Permanent Red)</td>
<td>Membranous (red signal) and nuclear (brown signal)</td>
</tr>
<tr>
<td>LANA-1 and DC-SIGNR</td>
<td>LANA-1 (DAB)</td>
<td>DC-SIGNR (Permanent Red)</td>
<td>Membranous (red signal) and nuclear (brown signal)</td>
</tr>
<tr>
<td>LANA-1 and p24</td>
<td>p24 (DAB)</td>
<td>LANA-1 (Permanent Red)</td>
<td>Nuclear (red signal) and membranous (brown signal)</td>
</tr>
<tr>
<td>LANA-1 and CD20 or CD3</td>
<td>LANA-1 (DAB)</td>
<td>CD20 or CD3 (Permanent Red)</td>
<td>Nuclear (brown signal) and membranous (red signal)</td>
</tr>
</tbody>
</table>

Positive LANA-1 staining was detected by a brown colour due to the chromogen used in all double staining immunohistochemical combinations, except LANA-1 and p24 (Table 3).

For the purposes of this study, the distribution (density as number of positive cells per 10 hpf) and intensity (either weak or strong) of HHV-8 LANA-1 immunohistochemical staining was specified by the investigators.

Weak staining has been defined as requiring examination under high magnification (x40 objective).

Strong staining has been defined as nuclear staining (multiple closely packed dots) visible at low magnification (x10 objective).
2.8 Immunohistochemical analysis

All 50 cases were assessed for:

- LANA-1 and DC-SIGN co-staining.
- LANA-1 and DC-SIGNR co-staining.
  - Co-staining cells were manually counted and their location within lymph node documented i.e. follicle, mantle zone or sinuses.

- LANA-1 positive cases were then scored for density of virally infected cells.
  - The total number of LANA-1 positive cells in 10 high power fields (HPF) was manually counted

- LANA-1 and p24 co-staining.
  - Co-staining cells were manually counted and their location within lymph node documented i.e. follicle, mantle zone or sinuses.

- LANA-1 and CD20 and CD3
  - Co-staining cells were manually counted and their location within lymph node documented i.e. follicles, parafollicular or paracortical zones.

All cases were assessed for concurrent Kaposi sarcoma.

2.9 Statistical analysis

Data used was determined by counting cellular staining patterns and quantifying by visual count on each slide per 10 high power fields.

Descriptive data was presented as mean, median and ranges for the entire cohort and for specific categories that included age, use of HAART, CD4 count and Kaposi sarcoma. Statistical analysis of quantitative variables was done using ANOVA. A significance level of \( P < 0.05 \) was deemed statistically significant for all statistical tests.

If the ANOVA analysis was significant, the Dunn's multiple comparisons test was performed to determine specific statistical significance between groups. Statistical analysis was done using STATA 14 and GraphPad Prism. Data was also visualised using
SAS (University Edition, Red hat, SA). Association between categorical variables was determined by using either a Chi squared test or a Fisher’s exact test as applicable. A significance level of $P < 0.05$ was deemed statistically significant for all statistical tests.
CHAPTER 3:  
RESULTS

3.1 Study cases

The specimen numbers of 65 cases of CD diagnosed over the study period were obtained from the LIS. Six cases that were diagnosed on needle core biopsies were excluded. 59 tissue blocks were then retrieved from the Anatomical Pathology archives. An additional 9 cases were later excluded due to damaged tissue blocks. A final total of 50 cases of CD were entered into the study. The number of cases diagnosed per year is shown in Figure 3.

![Figure 3: Number of cases of CD diagnosed at GSH from 2003 to 2013 (Jan-Jul)](image)

3.2 Morphological analysis

The 50 cases in the cohort were originally diagnosed as 25 (50%) HV-CD cases, 16 (32%) PC-CD cases and 9 (18%) mixed subtypes cases. None of the cases were originally diagnosed as plasmablastic subtype.
Figure 4: Castleman disease subtypes (A) Hyaline vascular (HV-CD) subtype with characteristic penetrating vessel. (B) Mixed-CD showing a combination of hyaline vascular and plasmacytic features. (C) Plasma cell (PC-CD) with plasmacytosis and occasional plasmablasts in the mantle zone (white arrows). (D) Plasmablastic (Pb-CD) with larger number of plasmablasts (40x obj magnification). (E) Kaposi sarcoma. (F) LANA-1 positivity in spindle cells (20x obj magnification).
The histopathology of all the cases was re-evaluated. Specific morphological criteria included assessment of germinal centre regression, increased vascularity, FDC prominence, interfollicular plasmacytosis and the presence of plasmablasts. On analysis, all cases satisfied the histopathological criteria for a diagnosis of CD, although there was some variation in morphological appearance. The cases were re-classified by the investigators using the plasmablastic-CD as an additional subtype after morphologic and immunohistochemical analysis (Figure 5). In total 29 cases were re-classified (Table 4 and Figure 8).

There was an observable increase in Pb-CD from 2006 (Figure 6 and Figure 7). After re-analysis of the entire cohort, there were equal numbers of HV-CD and Pb-CD subtypes (n=16) and equal numbers of PC-CD and mixed-CD subtypes (n=9) (Figure 9).

Figure 5: Density of LANA-1 staining (A) Hyaline vascular (HV-CD) subtype with characteristic penetrating vessels, atrophic hyalinised germinal centres, prominence of follicular dendritic cells and hypervascularity (B) Occasional LANA-1 positive cells (C) Plasmablastic (Pb-CD) with large numbers of plasmablasts with large vesicular nuclei and 1–2 prominent nucleoli (inset) (D) LANA-1 positivity in plasmablasts (arrow heads) (40x obj magnification)
According to the morphological criteria used, the two HV-CD cases that were re-classified as PC-CD showed a prominence of hyperplastic follicles, interfollicular plasmacytosis, presence of plasmablasts and an absence of distinctive vascularity. Four HV-CD cases
were further re-classified as Pb-CD after morphological analysis and immunohistochemical demonstration of LANA-1 positivity within these plasmablasts.

Table 4: Overview of reclassified CD cases

<table>
<thead>
<tr>
<th>Original number of Castleman disease</th>
<th>Number of reclassified cases</th>
<th>HV</th>
<th>PC</th>
<th>Pb</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV-CD n = 24</td>
<td>10</td>
<td>14</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>PC-CD n = 17</td>
<td>12</td>
<td>0</td>
<td>5</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Mixed-CD n = 9</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total number n = 50</td>
<td>29</td>
<td>16 (32%)</td>
<td>9 (18%)</td>
<td>16 (32%)</td>
<td>9 (18%)</td>
</tr>
</tbody>
</table>

Figure 8: Breakdown of original and reclassified CD subtypes
Figure 9: Number of CD subtypes before and after morphological reclassification

3.3 Age

The cohort of patients included 26 men and 24 women ranging in age from 14 to 76 years (median 36 years). The median age for the total cohort was 36 years (mean 37.7 years, range 14-76 years). HV-CD had a median age of 36.5 years (mean 37.8, range 14-76 years). PC-CD had a median age of 37 years (mean 38.6, range 23-61 years). Mixed CD had a median age of 33 years (mean 35.1, range 23-56 years). There were no statistically significant differences when comparing age amongst hyaline vascular, plasma cell, plasmablastic and mixed subtypes (Figure 10).
Figure 10: Age distribution amongst CD subtypes (including Pb-CD ♦)

3.4 Sex distribution
The cohort included 26 males and 24 female patients. This equates to a male: female ratio of 1.08:1. When assessing the sex ratio amongst the specific subtypes, the HV-CD was the only subtype that showed a female predominance (Table 5).

Table 5: Male:Female ratio amongst reclassified CD subtypes

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire cohort</td>
<td>26</td>
<td>24</td>
<td>1.08:1</td>
</tr>
<tr>
<td>HV-CD</td>
<td>6</td>
<td>10</td>
<td>0.6:1</td>
</tr>
<tr>
<td>PC-CD</td>
<td>6</td>
<td>3</td>
<td>2:1</td>
</tr>
<tr>
<td>Pb-CD</td>
<td>9</td>
<td>7</td>
<td>1.28:1</td>
</tr>
<tr>
<td>Mixed-CD</td>
<td>5</td>
<td>4</td>
<td>1.25:1</td>
</tr>
</tbody>
</table>

Summary

<table>
<thead>
<tr>
<th>Summary</th>
<th>Adjusted P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaline vascular vs. Mixed</td>
<td>ns</td>
</tr>
<tr>
<td>Hyaline vascular vs. Plasma cell</td>
<td>ns</td>
</tr>
<tr>
<td>Mixed vs. Plasma cell (including plasmablastic)</td>
<td>ns</td>
</tr>
</tbody>
</table>
3.5 Race
There were thirty-seven African (74%), eight Coloured (16%) and five White patients (10%) in the cohort.

3.6 Lymph node status
The lymph nodes were obtained from several different nodal regions. Twenty-seven (54%) patients presented with localised lymph node enlargement. Specified sites included 14 cervical (52%), 10 axillary (37%), 2 inguinal (7%) and 1 mediastinal (4%) lymph nodes. The location of the excised lymph node in the setting of generalised lymphadenopathy (46%) was not specified in all cases but included lymph node excisions from the axilla, neck and inguinal region. There was, however, not enough clinical information to definitively subclassify each case as either UCD or MCD.

3.7 HHV-8 status
HHV-8 LANA-1 immunohistochemistry was performed on only 37 cases at the time of diagnosis (32 positive and 5 negative). After LANA-1 immunohistochemistry was repeated on all 50 cases, LANA-1 positivity was present in the 32 cases initially reported as being positive, 3 of the 5 cases that were initially reported as being LANA-1 negative and 5 of the 13 cases that were previously untested. Immunohistochemical analysis revealed that 80% (n=40) of the cohort were LANA-1 positive and 20% (n=10) were LANA-1 negative (Figure 11).

Figure 11: LANA-1 immunohistochemistry results summary - original and revised
3.8 HIV status

HIV positivity is significantly associated with HHV-8 positivity in this cohort by Chi-squared test analysis. The Chi-square statistic is 22.22 (p-value = 0.000002) (Table 6).

Table 6: HIV and HHV-8 distribution of cohort

<table>
<thead>
<tr>
<th></th>
<th>HIV +</th>
<th>HIV -</th>
<th>Row Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-8 +</td>
<td>40* (36)** [0.44]***</td>
<td>0* (4)** [4]***</td>
<td>40</td>
</tr>
<tr>
<td>HHV-8 -</td>
<td>5* (9)** [1.78]***</td>
<td>5* (1)** [16]***</td>
<td>10</td>
</tr>
<tr>
<td>Column Totals</td>
<td>45</td>
<td>5</td>
<td>50 (Grand Total)</td>
</tr>
</tbody>
</table>

* number of observed cases; ** (number expected as per chi squared test); *** [chi squared statistic]

The cohort included 43 patients who were documented as being HIV positive (n=35) and HIV negative (n=8) using the clinical history provided on the requisition form or the recorded result of the ELISA test located on the DISA LIS, at the time of lymph node biopsy and diagnosis. The HIV-status was unknown in the remaining 7 patients. Six of the 35 HIV-positive patients had plasma HIV viral loads less assay detection (ie <50 copies/uL). HIV p24 IHC was performed on all the cases in the cohort. The original 35 reported HIV-positive cases, 3/8 of the reported HIV-negative cases and all 7 HIV-status unknown cases showed positive p24 immunohistochemical staining. After immunohistochemical evaluation of all 50 cases, 45 (90%) of the patients were confirmed as being HIV positive while 5 (10%) were HIV-negative (Table 6). A comparison of CD subtype with HIV and HHV-8 status showed a that there was a predominance of male Black patients with Pb-CD within the HIV(+)/HHV-8(+) subgroup (Table 7).
Table 7: Comparison of CD type with Age and Sex

<table>
<thead>
<tr>
<th>HIV and HHV-8 IHC status</th>
<th>HIV(+)/HHV-8(+)</th>
<th>HIV(+)/HHV-8(-)</th>
<th>HIV(-)/HHV-8(+)</th>
<th>HIV(-)/HHV-8(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>40</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>32</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Coloured</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>White</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>CD subtype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pb</td>
<td>Pb = 16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HV</td>
<td>HV = 7</td>
<td>5</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>PC</td>
<td>PC = 8</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mixed</td>
<td>M= 9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.9 CD4 counts and HAART

The CD4 count was known in 26 of the confirmed 45 HIV infected patients (including the p24 positive cases). There was no statistically significant difference in CD4 counts amongst CD subtypes (p = 0.1631) (Figure 12).
Multiple comparisons test | Summary | Adjusted P value
--- | --- | ---
Hyaline vascular type vs. Mixed type | ns | 0.9951
Hyaline vascular type vs. Plasma cell type | ns | 0.4985
Hyaline vascular type vs. Plasmablastic | ns | 0.6398
Mixed type vs. Plasma cell type | ns | 0.4427
Mixed type vs. Plasmablastic | ns | 0.6361
Plasma cell type vs. Plasmablastic | ns | 0.9883

Figure 12: Comparison of CD4 counts amongst subtypes (including Pb-CD♦)

The CD4 counts of the 26 patients ranged from 37 to 879 x 10⁶/l. The mean CD4 count was 270 x 10⁶/l (median 193 x 10⁶/l). Thirteen of the 26 cases had CD4 counts <200 x 10⁶/l (mean 119 x 10⁶/l, median 134 x 10⁶/l).

Twenty-two of the 26 patients with known CD4 counts received highly active antiretroviral therapy (HAART) prior to diagnosis of CD. There was no information regarding length of time between HAART initiation and disease presentation. It is undetermined as to whether the remaining 4 patients with known CD4 counts were
initiated on HAART after the diagnosis was made. Of the 22 patients already initiated on HAART, 14 (64%) (n=14) were classified as PC-CD (10 plasmablastic subtype), 3 (14%) as HV-CD and 5 (23%) as Mixed-CD. Six of the 22 patients enrolled on HAART had plasma HIV viral loads less than the level of detection of assay (<400 copies/ul, depending on the assay used), 6 patients had viral loads > 400 copies/ul, while the remaining 10 patients did not have documented viral loads. The remaining 9 patients of the original 35 patients who tested positive for HIV had neither documented CD4 counts nor clinical information regarding initiation of (HAART) prior to diagnosis.

3.10 Concurrent Kaposi sarcoma

Eight (16%) of the 50 cases (2 x HV-CD, 3 x PC-CD, 1 x Pb-CD and 2 x Mixed-CD) showed histological evidence of concurrent KS within the affected lymph node (Figure 13 and Figure 14).

Figure 13: Castleman disease and Kaposi sarcoma (A-B) Kaposi sarcoma within lymph node (C) Spindle cells and endothelial cells within KS (D) LANA-1 nuclear staining of the spindle cells (20x obj magnification)
Figure 14: Distribution of diagnosed CD subtypes with concurrent KS

3.11 Localisation of DC-SIGN and DC-SIGNR within lymph nodes

Cells expressing membranous DC-SIGN and DC-SIGNR were seen predominantly within the subcapsular sinuses, cortical sinuses, medullary sinuses and scattered within the paracortex (Figure 15).
Figure 15: Lymph node localisation of DC-SIGN and DC-SIGNR (A) Normal localisation of DC-SIGN expressing cells within subcapsular and cortical sinuses (B) Normal localisation of DC-SIGNR expressing cells seen predominantly within lymphatic sinuses

3.12 Localisation of p24 within lymph nodes

HIV infected cells with brown membranous/cytoplasmic staining for p24, showed almost exclusive localisation to the follicles (germinal centres and mantle zones) (Figure 16).


3.13 Location and density of LANA-1 positive cells amongst CD subtypes

When evaluated for distribution of HHV-8 infected cells, the HV-CD subtype (n=16) showed focal (≤10% of cells showing nuclear staining) and weak (discrete dot-like nuclear stippling seen at high magnification) LANA-1 immunohistochemical staining.

In contrast, when evaluated for HHV-8 infected cells the Pb-CD (n=16), PC-CD (n=9) and Mixed-CD (n=9) subtypes all showed a high density of cells showing dot-like nuclear staining (Figure 17 and Table 8).
Figure 17: Density of LANA-1 cells (A) HV-CD with low numbers of LANA-1 staining cells (20x obj magnification) (B) Pb-CD with numerous LANA-1 staining cells (20x obj magnification) (C) Strong LANA-1 dot-like nuclear staining of plasmablasts (40x obj magnification, insert under oil magnification)
Multiple comparisons test

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<tr>
<td>Plasma cell vs. Plasmablastic</td>
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Figure 18: Comparison of LANA-1 density amongst CD subtypes (including Pb CD ♦)

Table 8: Density of LANA-1 cells (per 10/hpf)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
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<td>HV-CD</td>
<td>9.96</td>
<td>5.95</td>
<td>19</td>
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<tr>
<td>PC-CD</td>
<td>44.6</td>
<td>34.6</td>
<td>101</td>
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<tr>
<td>Mixed-CD</td>
<td>47.4</td>
<td>44.7</td>
<td>129</td>
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<tr>
<td>Pb-CD</td>
<td>111.56</td>
<td>99.25</td>
<td>165</td>
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</table>

When comparing density of HHV-8 infected cells amongst the different subtypes, there was a statistically significant difference in density of HHV-8 infected cells between Pb-CD and all the other subtypes ie. HV, Mixed- and PC-CD all with p = 0.0002 (Figure 18).
3.14 LANA-1/DC-SIGN and LANA-1/DC-SIGNR

Figure 19: Co-localisation of LANA-1 and DC-SIGN/ DC-SIGNR within lymph nodes (A) Double immunohistochemical staining of Pb-CD subtype with LANA-1/DC-SIGN. LANA-1 (nuclear brown) and DC-SIGN (membranous red) co-expressing cells can be seen within sinuses (B) Co-localising LANA-1 and DC-SIGNR within lymph node sinuses (20x obj magnification)

All 50 of the CD cases were evaluated for HHV-8/DC-SIGN and HHV-8/DC-SIGNR co-expressing cells. The highest number of co-staining cells was observed in Pb-CD (mean = 7.9; median = 6; range = 4 - 20) with localisation of these cells predominantly within the sinuses (Figure 19). The least number of co-expressing cells were observed in HV-CD (mean = 1.6; median = 1.5; range = 0 – 4) (Table 9 and Table 10). There were 2 HV-CD cases that showed an absence of co-expressing cells.

Table 9: Number of LANA-1/DC-SIGN co-staining cells amongst CD types (per 10/hpf)

<table>
<thead>
<tr>
<th>CD Type</th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
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<tr>
<td>HV-CD</td>
<td>1.6</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>PC-CD</td>
<td>3.3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Mixed-CD</td>
<td>4.3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Pb-CD</td>
<td>7.9</td>
<td>6</td>
<td>16</td>
</tr>
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</table>
Table 10: Number of LANA-1/ DC-SIGNR co-staining cells amongst CD types (per 10/hpf)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
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<tbody>
<tr>
<td>HV-CD</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PC-CD</td>
<td>2.8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Mixed-CD</td>
<td>2.4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pb-CD</td>
<td>5.6</td>
<td>4</td>
<td>13</td>
</tr>
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The Pb-CD cases showed numerous LANA-1 positive cells within the subcapsular sinuses, medullary cords and intranodal sinuses with noticeable aggregation within follicles and germinal centres. It was predominantly within these specific locations that LANA-1/DC-SIGN and LANA-1/DC-SIGNR co-expressing cells were seen (Figure 19 and Figure 20).
Figure 20: Co-staining LANA-1/DC-SIGN (A) and DC-SIGNR (B). LANA-1 (nuclear brown) and DC-SIGN/DC-SIGNR (membranous red) co-expressing cells (40x obj magnification, inserts under oil magnification)
There was a statistically significant difference in the number of LANA-1/DC-SIGN co-staining cells between HV-CD and Pb-CD (p = 0.0072) and HV-CD and PC-CD (p = 0.0458) (Figure 21).

![Graph showing the comparison of co-staining LANA-1/DC-SIGN cells amongst CD subtypes](image)

<table>
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<th>Summary</th>
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Figure 21: Comparison of co-staining LANA-1/DC-SIGN cells amongst CD subtypes (including Pb-CD ♦)

### 3.15 LANA-1 and p24

Dual staining LANA-1/p24 cells were identified (Figure 22) with more of these cells seen in Pb-CD (Table 11). There was a statistically significant difference in the number of co-expressing cells between the Pb-CD and HV-CD (p < 0.0001) (Figure 23).
also statistical differences observed between HV-CD and Mixed-CD (p = 0.0007) as well as PC-CD and Pb-CD (p = 0.001) (Figure 23).

Figure 22: Double immunohistochemical staining with LANA-1 and p24. LANA-1 (nuclear red) and p24 (cytoplasmic brown) co-expressing cells (40x obj magnification and under oil)
Figure 23: Comparison of co-staining LANA-1 and p24 cells amongst CD subtypes (including Pb-CD ♦)

Table 11: Number of co-expressing LANA-1 and p24 cells (per 10/hpf)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
</tr>
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<tr>
<td>HV-CD</td>
<td>1.5</td>
<td>1</td>
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</tr>
<tr>
<td>PC-CD</td>
<td>3.6</td>
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</tr>
<tr>
<td>Mixed-CD</td>
<td>4.5</td>
<td>4.5</td>
<td>4</td>
</tr>
<tr>
<td>Pb-CD</td>
<td>7.9</td>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>
3.16 LANA-1 and CD20
Double immunohistochemistry using B-cell marker (CD20) and LANA-1 was performed on twenty cases in our cohort. Occasional co-expressing cells were seen, predominantly within the germinal centres and mantle zones (Figure 24) (mean = 3; median = 4; range = 4).

Figure 24: Co-staining LANA-1 and CD20 in Pb CD Double staining immunohistochemistry of Pb-CD subtype showing co-staining LANA-1 (nuclear brown) and CD20 (cytoplasmic red) cells located predominantly within the follicles (20x obj magnification, insert 40x obj magnification under oil).

3.17 LANA-1 and CD3
Double immunohistochemistry using T-cell marker (CD3) and LANA-1 was performed on twenty cases in our cohort. There were no observable cells that demonstrated double staining immunoreactivity.

3.18 LANA-1 negative CD
Interestingly, the 5 cases that were both p24 and LANA-1 negative after immunohistochemical analysis were all morphologically classified as HV-CD by the
investigators (Figure 25). There were also 5 cases that were LANA-1 negative but showed p24 immunoreactivity.

After LANA-1 and p24 immunohistochemical analysis, a total of 10 patients were found to be HHV-8 negative. There were 5 HIV negative patients and 5 HIV positive patients. The HIV-negative/HHV-8-negative patients showed a female predominance (M:F 2:3) and a sex ratio of 0.7:1. There were 3 white (female) patients, 1 coloured male and 1 African male. Interestingly all 5 cases were classified as HV-CD based on lymph node histomorphology.

The HIV-positive/HHV-8 negative patients showed a male predominance (M:F 3:2) and sex ratio of 1.5:1. There were 4 African patients (M:F 2:2) and 1 coloured male patient. There were 4 cases that were classified as HV-CD and the remaining case as PC-CD. Overall, 90% (n=9) of the HHV-8 negative patients were classified as HV-CD.

Figure 25: LANA-1 negative CD. HV-CD showing no LANA-1 staining (40x obj magnification)
CHAPTER 4:
DISCUSSION

The current study represents the largest series of Castleman disease investigated in a South African setting and it is, to my knowledge, the first immunophenotypic study to use double-staining immunohistochemistry to elucidate the pathophysiology and biology of HHV-8 infected cells in a Western Cape CD cohort. The previous largest series was published by Patel et al. in 2015 and included 35 cases seen over a period of 25 years.

The study cohort included 26 male and 24 female patients (M: F = 1.08:1), mean age 37.7 years. There were 16 HV-CD, 16 Pb-CD, 9 PC-CD and 9 mixed-CD subtypes. There was an increase in number of patients diagnosed with HIV associated CD over the study period, and an increase in the number of cases classified as Pb-CD subtype. There was a statistically significant association between HIV and HHV-8 positivity (p < 0.0002). CD4 counts and HAART enrolment were not predictive of CD development (p = 0.6120). Concurrent Kaposi sarcoma was seen in 16% (n=8) of the cohort. When comparing Pb-CD and HV-CD, there were statistically significant differences in density of LANA-1 infected cells (p<0.0002), LANA-1/DC-SIGN co-expressing cells (p <0.0072) and LANA-1/p24 co-expressing cells (p<0.0001). LANA-1/DC-SIGN co-expressing cells were seen in the subcapsular sinuses, intranodal sinuses and medullary cords while LANA-1/DC-SIGNR co-expressing cells were seen within intranodal sinuses. The majority LANA-1/p24 co-expressing cells were localised within the parafollicular and mantle zones of the lymph nodes. LANA-1/CD20 co-expressing cells were seen within the follicles and parafollicular zones.

The clinical and pathological manifestations of the CD subtypes have previously been described in several other small case series (Keller et al., 1972; Frizzera, 1988; Herrada et al., 1998; Mylona et al., 2008; Bower et al., 2011). Since 2011, there have been publications of larger case series reporting the epidemiology and natural history of CD (Dispenzieri et al., 2012; Casper et al., 2013). There have also been a few studies that focused on variation of patient subsets within defined CD histological subgroups, HIV status and ethnicity (Mylona et al, 2008; Stebbing et al, 2008).
4.1 Incidence and prevalence

Despite the increasing awareness of the disease among both physicians and pathologists, the exact incidence and prevalence of CD, especially HIV-associated HIV-associated MCD, is not yet known in South Africa or sub-Saharan Africa. Consequently, we are not able to establish the actual burden of disease. This can be partially attributed to the lack of clinicopathological studies coming out of South Africa. The fact that there are also no diagnostic/ICD-10 codes that currently exist for CD, UCD or MCD, highlights the rarity of CD while at the same time emphasises the need for more recognition of this disease. In countries like the USA where there has been a surge in interest regarding CD, large patient studies have estimated that the annual incidence is in the region of 6500 cases (Munshi et al., 2015).

Not only does our cohort show a substantial increase in the frequency of CD diagnosed at Groote Schuur hospital during the study period, it also highlights the rising incidence of plasmablastic CD since 2006. These findings are to be expected given the high prevalence of both HIV and HHV-8 infection in our population. Although Pb-CD is known to occur with increased frequency in the context of HIV and AIDS, with a few international publications addressing its incidence in countries such as the USA and UK (Gerard et al., 2007; Powles et al., 2009), there is a distinct absence of regional South African statistics. The lack of specific data in South Africa may be related to the fact that CD is still a relatively rare disease and therefore not as yet recorded on the National Cancer Registry (NCR). HHV-8 seroprevalence is reported to be high in sub-Saharan Africa compared to other regions prevalence rates.

In South Africa, a study conducted at an HIV clinic in Johannesburg found a high HHV-8 prevalence of 48% (Maskew et al., 2011) which correlated with another South African study that indicated the HHV-8 seroprevalence varied from 35% to 49% across different municipalities in one province (Malope-Kgokong et al., 2010). A study conducted in the Western Cape province of South Africa found the HHV-8 seroprevalence to be 45.45% in HIV infected patients without KS (Blumenthal, 2017).

Although, HIV prevalence remains high (12.7%) nationally among the general population, there is also interprovincial variation with high prevalence and incidence rates in Gauteng and Kwazulu Natal compared low HIV prevalence in Northern Cape and
Western Cape (South African National AIDS Council, 2012). Larger studies will therefore be of great value in obtaining prevalence and incidence rates of CD, both provincially and nationally, as there have been no comprehensive studies to date.

4.2 Patient demographics

The mean patient age for the entire cohort was 37 years (median age = 36 years and range = 14 – 76 years). The age range for HV-CD was 14 – 76 years, the majority of cases occurring between 20-45 years with a mean age of 37.8 years and median of 36 years. This is similar to the findings described by Mylona et al. (2008), who reported that unicentric HV-CD most commonly presents in the third and fourth decade of life, with the mean age of diagnosis being 34 years. The age range for the PC-CD (including the plasmablastic subtype) was 23 – 61 years, with a median of 37 years and mean age of 38.6 years. HIV-associated MCD was found to affect mainly men with median age of 40 years. Other studies observed that HIV-positive patients had a younger age of presentation in comparison to HIV-negative patients (Dispenzieri, 2011; Mylona et al., 2008; Bower et al., 2011). The median age of onset of HIV-negative individuals was 56 years (Fagenbaum et al., 2014). This correlated with our findings which showed a median age of 37.7 years for HIV positive patients and 52 years for HIV negative patients.

The male to female ratio in this study was 1.08:1. There is, however, variation in the literature regarding overall sex distribution. Although Soumerai et al. (2014) observed a slight male predominance, which aligned with our cohort analysis, this finding differed when compared to a study by Bower et al. (2011) and a systemic review by Mylona et al. (2008), where a significant male predominance was reported (87% and 90% respectively).

According to Casper (2005), there is no observable predilection for either sex with regards to the HV-CD. This differed to our cohort study which showed a predominance of females (62.5%) within the HV-CD group (M:F = 0.6:1). This finding correlates more closely with a study by Talat et al. (2012) which showed that HV-CD had a slight female predominance (1.4:1). HIV-associated PC-CD, meanwhile, is found to mainly affect men (Mylona et al., 2008) which differed from our cohort (M:F = 1.3:1), which only showed a slight male predominence. This discrepancy might be explained in part by the fact that
females, represent a larger proportion of the HIV population in South Africa than in other part of the world (Mosam et al., 2008). The sex ratio of the entire cohort is 1.08:1, while the sex ratio for HIV/HHV-8-associated CD is 1.10:1. This finding underscores the shift in previously high male HHV-8 seroprevalence rates, to females (Sitas & Newton, 2000; Stein, 2008; De Cock et al., 2012). This variation of sex predominance in the literature implies that CD epidemiology is still poorly understood and highlights the fact that more studies are required with regards to sex distribution.

Larger studies are required to determine true prevalence and incidence of CD. Knowing the burden of disease and counts of the number of patients affected with CD is required to plan appropriately for health care and oncology related services. Prevalence estimates, are also useful clinically by providing context for diagnostic decision-making in clinics and may be helpful in comparing disease burden across different provinces in South Africa, sub-Saharan Africa and the rest of the world. Accurate incident rates are very important as it would assist in disease aetiology, by comparing how the rates vary among different subgroups in South Africa or with different exposures to HHV-8.

With uncommon diseases, such as CD, there are understandable challenges because cases are fewer and harder to find. These factors definitely necessitate surveys of even larger populations to achieve stable estimates (as well as longer durations of observation for estimates of incidence), which in turn increase the cost, time, and effort involved in executing such studies. Because of these issues, studies using primary data collection to determine the prevalence and incidence of diseases such as CD are not common.

4.3 Anatomical location
Although the first case series reporting occurrences of CD specified the mediastinum as the most frequently involved site for lymph node enlargement, subsequent series have found cervical, abdominal and axillary lymphadenopathy to be equally common (Castleman et al., 1956; Keller et al., 1972; Herrada et al., 1998). One study has documented predominantly mediastinal (60–75%), cervical (20%) and abdominal (10%) masses (Bower, 2010) while a subsequent study by Bower et al. (2011) showed axillary (88%), abdominal (78%), pelvic (70%), mediastinal (58%) and cervical (35%) lymphadenopathy. In our cohort, there were 27 (54%) unicentric and 23 (46%)

73
multicentric presentations with a preponderance of cervical (52%) and axillary (37%) lymphadenopathy observed in UCD. There was insufficient clinical data to accurately link anatomical location with histological CD types.

4.4 Unicentric and Multicentric CD

Although multicentric CD was initially separated from unicentric CD by its multifocal nature, it is now clear that it represents a different disease with different pathogenesis and much worse clinical outcome. Virtually all MCD cases in HIV-positive patients are associated with HHV-8, although HHV-8 positive MCD has also been known to occur, albeit rarely, in HIV-negative patients.

The hyaline vascular subtype was found in 90% of UCD patients but rarely in those with MCD, while the plasma subtype was found in only 10% of UCD patients but in 80–90% of those with MCD (Waterston & Bower, 2004; Casper, 2005; Dispenzieri, 2012; El-Osta & Kurzrock, 2011). Unicentric PC-CD, which accounts for <20% of all CD subtypes, is usually characterized by an aggregate of multiple, discretely enlarged nodes within a single lymph node chain and is characterised by sheets of polytypic plasma cells within the interfollicular zones. The follicles are usually of normal to large size with hyperplastic germinal centres and showed no prominent vascularization or hyalinization (Cronin and Warne; 2009). After reclassification, 18% (n=9) of the cases were PC-CD. Furthermore, PC-CD cases that showed increased numbers of LANA-1 positive plasmablasts were additionally subtyped as Pb-CD. These plasmablasts were localised in the mantle and marginal zones and were characterised by amphophilic cytoplasm and large nuclei with one or two prominent nucleoli similar to findings by other researchers (Dupin et al., 2000; Brousset et al., 2001; Chadburn et al., 2008). Eventually, 32% (n=16) of our cases were classified as Pb-CD. This finding in our study highlights the rising incidence of HIV/HHV-8 associated CD in our patient population.

Plasmablastic CD is currently considered a distinct subtype defined by the unique population of HHV-8 infected plasmablasts within the lymph nodes of HIV-infected patients. (Dupin et al. 2000). Previous immunohistochemical studies have already characterised these HHV-8 infected plasmablasts with the accepted consensus that these
cells are in fact B-cell derived and are characterized by CD20+/-, CD19+, CD27+, CD38+/-, IgM+ and vIL6+ expression (Du et al., 2001).

Although these cells have characteristic cytomorphological features, virally infected cells are difficult to identify on morphology alone. As LANA-1 is the only protein whose expression is stably and consistently detected by immunohistochemistry in HHV-8 infected cells, the presence of strong dot-like nuclear positivity in these plasmablasts, even at low magnification, allowed for quick identification of these cells in our cohort (Dupin et al., 1999; Dupin et al., 2000). Although there is no consensus on the specific number or percentage of virally infected plasmablasts that is required for diagnosis, some researchers have stated that the number of plasmablasts ranged from 1% to 15% of the total population of cells in affected lymph nodes and spleens (Du et al., 2001). These researchers went on to further demonstrate that cases of plasmablastic lymphoma were composed of confluent sheets of plasmablasts with identical immunophenotypic features to those of HHV-8 positive mantle zone plasmablasts and microlymphomas.

Although some of our plasmablastic cases showed occasional aggregates and clusters of plasmablasts within the mantle zones, none of our cases showed features of “microlymphoma” or features suggestive of frank lymphomatous progression. This finding is significant as plasmablastic CD, microlymphoma and HHV-8+ plasmablastic lymphoma are postulated to represent three stages of a single disease in a background of HIV-induced immunodeficiency (Cronin and Warnke., 2009). The recent 2015 Workshop of The Society for Hematopathology/European Association for Haematopathology recently held a workshop that aimed to review immunodeficiency-related lymphoproliferations with plasmablastic and plasma cell differentiation (Chadburn et al., 2017). One of the conclusions of this workshop was that HHV-8 presence or absence is a defining feature in disorders associated with CD and that recognising disease progression can be difficult.

4.5 HHV-8 positive CD
There is geographic variation in the prevalence of HHV-8 seropositivity with more than 50% seropositivity in sub-Saharan Africa, 20-30% seropositivity in Mediterranean
countries and less than 10% in Europe, Asia and USA (Uldrick & Whitby, 2011). There were 32 black patients, 6 coloured and 2 white patients.

In regions with high HHV-8 seroprevalence in the general population, there is an equally high burden of disease. Two studies based in South Africa revealed an HHV-8 seroprevalence of 32% and 34.5% (Wilkinson et al., 2002; Sitas et al., 1999). The prevalence of HHV-8 antibodies increased with increasing age, increased number of sexual partners and decreased with increasing levels of education. In addition, HHV-8 serum antibodies were more prevalent in Black patients compared to White patients (Sitas et al., 1999).

Cross sectional studies aiming to define the seroprevalence of HHV-8 in South African populations were undertaken by Malope et al. (2012). They found that HHV-8 seroprevalence was significantly higher in HIV-infected subjects (P = 0.0005), and that HIV-infected subjects had significantly higher lytic and latent HHV-8 antibody levels than HIV-negative subjects.

It has already been well established that HHV-8 plays a major pathogenic role in the development of HIV-associated MCD. HIV, in addition to its own pathogenic mechanisms, has direct effects on HHV-8 by promoting re-activation and replication of latent HHV-8 (Merat et al., 2002). Currently, it is known that HHV-8 is present in 100% of MCD in patients infected with HIV and in only 40% to 50% of HIV-negative cases (Soulier et al., 1995; Du et al., 2001). This was reflected in our study with 40 (90%) of our cases showing LANA-1 positivity while 10 cases were LANA-1 negative (5 HIV positive and 5 HIV negative). None of our patients were LANA-1 positive/HIV-negative. LANA-1 immunoreactivity was demonstrated on 3 cases previously reported as being LANA-1 negative. This discrepancy could be attributed to the fact that the original diagnostic stains were done on an automated platform, while manual staining was performed on the research slides for this study.

One of the interesting findings not previously described in the current literature is the statistically significant difference in density of HHV-8 infected cells between Pb-CD and each of the other CD types (p = 0.0002). This supports studies by Schulz (2006), who demonstrated that up to 50% of affected cells within the mantle zones of plasmablastic CD
lymph nodes can show positive LANA-1 staining (Schulz, 2006). This finding may also imply that that Pb-CD patients have higher HHV-8 viral copy numbers, higher HIV viral loads and lower CD4 T-cell counts.

Interestingly there was a statistically significant difference in density observed between the HV-CD and mixed-CD \((p = 0.0291)\) but no statistically significant differences observed between HV-CD and PC-CD \((p = 0.0584)\) or mixed-CD and PC-CD.

### 4.5.1 HIV status

The pathogenic role of HHV-8 is enhanced by HIV-induced immunosuppression. The rising incidence of MCD could be attributed to the increased awareness of this disease amongst health care professionals or due to the increased number of biopsies and lymph node excisions undertaken in clinical scenarios of lymphadenopathy with inflammatory symptoms where the disease is suspected.

Maskew et al (2011) confirmed an HHV-8 seroprevalence of 48% in their study of 440 HIV positive patients based in a Johannesburg clinic. The multivariate analysis of patients with HIV-associated MCD by Powles et al (2009), showed that the incidence is increasing in the HAART era. Further to this, it was also found that patients with well-preserved immune function, increased age and short retroviral-status duration predisposed to the development of MCD which was in contrast to the factors that predisposed to KS (Powles et al., 2009). This data therefore suggests that although both MCD and KS are associated with HHV-8, an immunosuppressive state predisposed to KS but not necessarily MCD. These investigators hypothesised that the biology of HHV-8 was altered in MCD thereby causing a dysregulated immune response resulting in the inflammatory cytokine cascade often seen in MCD, which would imply that there was relatively well-preserved or increasing immune function in the HAART era.

Previous investigations have shown that more lytic viral proteins rather than latent proteins are expressed in MCD compared to KS (Marcelin et al., 2007). Powles et al (2009) therefore speculated that the role of HHV-8 in the pathogenesis of MCD is most likely influenced by a well preserved immune system as opposed to the course of disease observed in KS patients.
Our cohort showed that 45 (90%) cases were HIV positive, when re-evaluated for p24 immunohistochemistry. We were unable to ascertain if the HIV tests done previously were HIV-1/2 Ab/Ag ELISA or rapid onsite finger prick tests used at primary health care facilities. After p24 immunohistochemistry analysis, 3 of the 5 patients that were originally reported as being HIV negative (ELISA), showed nodal p24 immunoreactivity. This discrepancy between ELISA and p24 IHC may be due to the fact that p24 concentration are often too low between initial infection and detection (window period) by the conventional ELISA (Laperche et al., 2012). A literature search looking for reports of false positive p24 immunoreactivity within lymph nodes did not reveal any results.

Moonim et al (2010) immunohistochemically evaluated HIV-1 p24 in 123 cases over a 3-year period, where 37 (30%) cases showed positive expression of p24 in follicular dendritic cells (FDCs) with 11 of these 37 cases neither clinically suspected to be HIV positive nor having any prior serological evidence of HIV infection (Moonim et al., 2010).

The presence of HHV-8 in all of our patients with HIV associated CD may be due to an HIV-induced cytokine rich microenvironment that is particularly favourable to HHV-8 replication. Dupin et al (2000) attributed such a finding to unhindered proliferation of HHV-8 in HIV-positive patients, which would ordinarily be more suppressed in immunologically competent individuals. Due to the strong association of HHV-8 MCD with HIV infection in our South African sample, we therefore propose that the diagnosis of Pb-CD be highly suggestive of immunosuppression in an individual with an otherwise unknown immune status.

All 25 patients reclassified with PC-CD (n=9) and Pb-CD (n=16) were HIV positive, which correlated with studies that reported that HIV-associated CD is almost always either PC-CD or mixed CD (Oksenhendler et al., 1996; Herrada et al., 1998; Mylona et al., 2008; Powles et al., 2009; Bower et al., 2011; Soumerai et al., 2014). More importantly, our study observed an increase in the plasmablastic subtype which is most likely driven by the presence and increase in latent gene proliferation of HHV-8 within plasmablastic cells, as postulated by Dupin and colleagues (2000).
Of the 16 patients with HV-CD, 11 (69%) were HIV infected, which differs from other case series that found that HV-CD usually shows no association with HIV infection (Mylona et al., 2008; Soumerai et al., 2014). It is possible that our study findings are more reflective of the high HIV seroprevalence in our population.

HAART did not prevent the development of CD as 22 of our 45 HIV infected patients were enrolled on HAART at the time of diagnosis. Although there is an increased incidence of MCD in HIV-positive patients who have low CD4 counts, the documented CD4 counts in our patient cohort ranged from 37 to 879 x 10⁶/l (mean = 270 x 10⁶/l, median = 193 x 10⁶/l). There was no correlation between the occurrence of CD and CD4 counts in our cohort (p=0.1631). These findings are similar to epidemiological studies which found that neither low CD4 counts nor use of HAART was associated with development of CD (Mylona et al., 2008; Bower et al., 2011). It is therefore possible that the occurrence of CD after initiation of HAART could be part of an immune reconstitution syndrome.

Some studies have even found that the incidence of HIV-associated MCD appears to be increasing even in the HAART era (Stebbings et al., 2008). This is in contrast to the decline in the incidence of HIV-associated KS (Mylona et al., 2008). The exact mechanism for this increase is unclear, but may possibly be due to improved survival rates, chronic immune dysregulation caused by HIV infection or even chronic immune activation by HAART.

4.5.2 Co-existent Kaposi Sarcoma

Based on consistent detection of the HHV-8 genome in both KS and MCD in conjunction with frequent co-presentations in the same specimen, it is now widely accepted that HHV-8 plays a critical role in the pathogenesis of both these diseases and that there is in fact, an increased incidence of KS in individuals with HHV-8 associated CD (Powles et al., 2009). PCR analysis shows that HHV-8 is present in all epidemiologic types of KS i.e. AIDS-associated KS, classic KS, endemic KS, and post-transplant KS (Moore & Chang, 1998). The HIV prevalence in South Africa in 2016 was reported as 12.7% with 18.9% of population between the ages of 15-49 years being affected (STATSSA). HHV-8 seroprevalence in the HIV population is between 44% and 48% (Malope et al., 2008;
Maskew et al., 2011) with an almost 50-fold increase in risk for developing KS (Stein, 2008).

HAART is effective on KS as seen by the regression of KS in individual cases and a dramatic fall in overall incidence which can be attributed to improving immune function with HAART use (Powles et al., 2009). Data also suggests that KS is dependent on the host’s immune status and CD4 counts (Bower et al., 2009). KS should be diagnosed with both histology and immunohistochemistry. Anti-LANA-1 immunohistochemical staining with characteristic nuclear speckled staining of spindle cells, shows that the viral protein is expressed in KS cells, regardless of clinical type or disease stage (Dupin et al., 1999). PCR analysis can even be performed on formalin-fixed paraffin-embedded KS tissue to confirm diagnosis based on the identification of HHV-8 DNA fragments in the tissue and occasionally in the sera of KS patients (Asahi-Ozaki et al., 2006).

Eight cases (16%) of concurrent KS and CD were identified in our cohort of 50 CD cases. The microscopic diagnosis of KS in our study was confirmed by nuclear positivity for the LANA-1 in KS cells within the lymph node capsule, subcapsular sinuses and hilum. The presence of these focal subcapsular spindle cell proliferations has been attributed to the virus remaining localised within the subcapsular sinuses, as suggested by O’Leary et al (2010). Although the origin of KS cells is thought to be endothelial cells, the protein expression of KS cells is very different from that observed in vascular endothelial cells as HHV-8 induces an increase in lymphatic endothelial markers (Hong et al., 2004). This was also observed in study by Dupin et al (1999) who showed the similar distribution of VEGFR-3, a lymphatic marker, and HHV-8 in both early and late KS lesions.

All of our cases with KS showed features of early KS consisting of microscopic foci of KS spindle cells within the capsule and hilum as opposed to advanced KS which often effaces most of the lymph node architecture. This emphasises that these foci can easily be missed in routine histological evaluation or even after immunohistochemistry.

As LANA-1 is expressed in the nucleus of KS cells and is responsible for establishing and maintaining latency, it can therefore be reasoned that latent infection is important for the pathogenesis of KS infection. Our cohort was too small to correlate with published findings which showed that patients with HIV-associated CD have a 54% to 72% risk of
being diagnosed with KS, either concurrently or sequentially and that the two diseases may coexist in the same pathological specimen (Mylona et al., 2008; Naresh et al., 2008). Although lytic gene expression is limited in KS, HHV-8 encoded lytic proteins such as vIL-6 which interacts with the human IL-6 receptor and partially mimics its function, are important for KS pathogenesis and can be detected at high levels in KS patient’s sera (Liu et al., 1997). vIL-6 can also be detected at high levels in the sera of MCD patients (Parravicini et al., 1997). It is the elaboration of vIL-6 in KS, which also causes a proliferation of plasma cells and increased angiogenesis, can eventually contribute to the concurrent development of CD in a setting of HHV-8 infection. This interaction may also explain the formation of LANA-1 positive KS ‘tumourlets’ within a background of CD (O’Leary et al., 2010).

Other studies that have also investigated the association of these two entities postulated that it was most likely due to both latent and lytic infection of B-cells by HHV-8 in MCD, which in turn leads to a wide variety of susceptible cells within the lymph node being exposed to very high levels of the virus (Abe et al., 2006; Naresh et al., 2008).

Apart from the latent and lytic genes that are expressed by HHV-8, studies have also identified miRNAs that have been shown to affect KS tumour biology by modulating host gene expression profiles (Samols et al., 2007). None of our HIV-negative patients showed evidence of concurrent KS which correlated with studies that showed that the association of KS is lower with HIV-negative MCD patients, where the reported dual incidence is between 0% and 13% (Mylona et al., 2008). This is in contrast to iMCD which shows no association with either virus.

4.5.3 Dual staining LANA-1 and DC SIGN/DC-SIGNR
HHV-8 infection of target cells involves both multiple host cell surface receptors and viral envelope glycoproteins. DC-SIGN has emerged as a key player with regards to HHV-8 infection of monocytes, MDDCs, monocyte-derived macrophages, B-cells, Langerhans cells, interstitial cells, and plasmacytoid dendritic cells (Rappocciolo et al., 2006; Rappocciolo et al., 2008; Knowlton et al. 2014). The identification of co-expressing LANA-1 and DC-SIGN/DC-SIGNR in our study correlates with Hensler et al. (2014) who have already previously demonstrated that HHV-8 glycoprotein B serves as a viral attachment protein responsible for binding to cellular receptor DC-SIGN.
In our study, DC-SIGN expressing cells were located in paracortical sinuses, within medullary cords and along the capsule and trabeculae of lymph nodes. Cells expressing a low level of DC-SIGN and DC-SIGNR were also scattered in the sinuses, the medullary cords and in the perifollicular zone of the paracortex. The above findings can be accounted for by the fact that although DC-SIGN expressing cells are present in all lymphoid organs, in lymph nodes the majority of these dendritic cells are located near the entry points for lymph, specifically in the cortical sinuses, whereas most DCs in the paracortex fail to express DC-SIGN. (Soilleux, 2003). This, coupled with the expression of DC-SIGNR by very restricted subsets of endothelial cells, may explain the localisation for co-expressing cells seen in our study.

Although the exact mechanism of how HHV-8 travels to and infects cells within lymph nodes is not completely understood, Campbell et al (2014) have postulated that after infection of the mucosal epithelium by HHV-8, the virus most likely infects blood and tissue DC-SIGN expressing monocytes and macrophages to gain access into the draining lymphoid organs where it then interacts with resident B and T cells. We were able to clearly demonstrate this distribution of HHV-8 infected DC-SIGN/DC-SIGNR expressing cells from the periphery of the lymph node (sinuses) into the follicles.

Several investigations have also found HHV-8-infected B-cells in the mantle zones of lymph nodes from patients with KS, MCD and nodal PEL (Dupin et al., 1999). This would therefore imply that DC-SIGN is also expressed within the parafollicular zones of lymph node tissue. Although our particular study has not demonstrated co-expression of HHV-8 and DC-SIGN in B-cells, other investigators have already done so (Rappociolo et al., 2006). These cells may serve as a source of latently infected B-cells that can potentially undergo oncogenic transformation and subsequently increasing the risk of HHV-8-related lymphoma development.

LANA-1 is not detectable in follicular dendritic cells (FDC) within lymph nodes isolated from KS, PEL, and MCD patients (Dupin et al., 1999). Other authors, in contrast, found that a proportion of FDCs expressed LANA-1 which was associated with an enhanced T-cell response and increased T-cell infiltration into lymphoid organs (El-Daly et al., 2010).
In our study, co-staining LANA-1/DC-SIGN and LANA-1/DC-SIGNR cells were only occasionally seen. The low numbers of these co-expressing cells may be attributed to the findings that HHV-8 infection of MDDCs, SMDCs and blood circulating monocytes is associated with a decrease in DC-SIGN expression (Rappocciolo et al., 2006). Interestingly, it has been suggested that K3 and K5, both HHV-8 encoded proteins, have the ability to decrease levels of DC-SIGN and DC-SIGNR on the surface of virus-infected kidney cells (Lang et al., 2013), but it has yet to be revealed if LANA-1 functions in this manner in DCs within affected lymph nodes. Available data also suggests that DC-SIGN is positively regulated in response to extracellular pathogen-based immune responses and is negatively regulated in response to intracellular pathogen-based responses such as viral infections (Rellosa et al., 2002; Rappocciolo et al., 2006). This theoretically could explain the fewer than expected numbers of HHV-8 infected dendritic cells in our study.

Despite the small number of co-staining cells observed in our study, there was a statistically significant difference in the number of LANA-1/DC-SIGN and LANA-1/DC-SIGNR co-expressing cells observed between HV-CD and Pb-CD (p = 0.0072). There was also a statistically significant finding between HV-CD and PC-CD (p = 0.0458). There were no other statistically significant findings between the other CD types.

The presence of co-staining cells seen in the vicinity of sinuses and surrounding medullary cords can be attributed to lymphatic homing receptors, such as CCR which are up-regulated, to attract them to CCL19 and CCL21 expressing high endothelial venules (HEVs), which triggers the migration of DCs into lymphatic vessels and to draining lymph nodes (Holmgren and Czerkinsky, 2005). We were also able to observe the distribution of co-localising cells within the subcapsular and intranodal sinuses, perivenular zones, follicles and germinal centres where they subsequently came into contact with naïve B-cells.

HHV-8 is one of the few human viruses that primarily targets APCs, monocytes/macrophages and polyfunctional B lymphocytes for infection, altering their cytokine profiles, manipulating their surface expression of MHC molecules and altering their ability to activate HHV-8-specific T-cells (Knowlton et al., 2014). Our findings are of importance as it further solidifies evidence that DC-SIGN serves as an entry receptor for HHV-8. This is also supported by the fact that most of the major cell types that can be
infected with HHV-8, express DC-SIGN and that infection of these cells can potentially be blocked by anti-DC-SIGN antibodies or natural ligands for DC-SIGN. This was also demonstrated in a study Rappocciolo et al. (2008) where active infection of blood and tonsillar B-cells expressing DC-SIGN by HHV-8 could be blocked with the use of anti-DC-SIGN monoclonal antibody. Hence DC-SIGN and the closely related C-type lectin DC-SIGNR, may provide the key to the development anti-viral therapies designed to prevent HHV-8 infection and spread.

4.5.4 Dual staining LANA-1 and p24
We already know that HHV-8 seroprevalence is higher in HIV-infected compared to HIV-uninfected population. Viral co-infection, with associated increased cellular proliferation and transformation, often represents a common mechanism in the pathogenesis of malignancies especially affecting immune compromised hosts.

The simultaneous detection of co-infecting viruses within the same neoplastic cells is seen in other HIV related malignancies such as HIV-associated PEL cells harbouring EBV and HHV-8 viruses (da Silva & de Oliveira, 2011), and implies viral co-operation in oncogenesis and subsequently lymphomagenesis. HIV represents one of the main viruses that cooperates with other viruses in the induction of cancers.

In our study, LANA-1/DC-SIGN co-expressing cells were also seen within the lymph node sinuses which is supported by the findings of Casselli et al. (2005) that the presence of HHV-8 activates HIV replication during macrophage passage through the endothelial barrier. As a result of this circulating monocytes produce significantly higher amounts of HIV in the presence of HHV-8. This was also illustrated by our cohort with a statistically significant association between HIV and HHV-8 infection (p-value = 0.000002). Although there has been intensive research done on HIV and HHV-8 cellular targets, the identification of co-staining LANA-1/p24 cells in CD in our study is a result not previously described. Previous studies have shown that DC-SIGN binds host ICAM-2/3 as well as HIV gp120 ligands on separate sites of the DC and is able to transmit the virus to activated B-lymphocytes (Geijtenbeek et al., 2002; Su et al., 2003). Closely related DC-SIGNR, although expressed by restricted subsets of endothelial cells, has similar ICAM-3 and HIV-binding properties to DC-SIGN (Soilleux, 2003).
We know that p24 immunoreactivity localises to follicular dendritic cells due to the presence of transmissible HIV particles on the surface of FDCs which are in very close contact with surrounding T- and B-cells. We can therefore surmise that the co-staining LANA-1/p24 cells in our study are follicular dendritic cells. This conclusion is supported by El-Daly et al (2010) who demonstrated that LANA-1 was present on follicular dendritic cells in a proportion of MCD cases. Furthermore, it was also shown that the LANA-1 positive FDC subgroup of patients had significantly higher numbers of T cells within the follicles in comparison to the LANA-1 negative FDC subgroup (p = 0.047).

It has also been demonstrated that HIV is internalised by B-lymphocytes after binding DC-SIGN (Rappocciolo et al., 2006). The same investigators then demonstrated that HHV-8 infects and replicates in activated B-lymphocytes via DC-SIGN (Rappocciolo et al., 2008). Using this data, we can also therefore assume that some of the cells that express both LANA-1 and p24 in our study are most likely to be co-infected B-lymphocytes. This raises many questions regarding the important role that FDCs play in the transmission kinetics of both HIV and HHV-8. FDCs unlike other dendritic cells do not express DC-SIGN or DC-SIGNR which prevents internalisation of HIV thereby making transmission to adjacent T- and B-cells all the more effective (Keele et al., 2008). Interestingly, El-Daly (2010) also found that the LANA-1 positive FDC subgroup had fewer numbers of LANA-1 infected plasmablasts and lower HHV-8 viral copy numbers than the LANA-1 negative FDC subgroup (not statistically significant).

Our Pb-CD had statistically significantly higher numbers of LANA-1 infected plasmablasts, LANA-1/DC-SIGN, LANA-1/DC-SIGNR and LANA-1/p24 co-staining cells when compared to the other CD subtypes. It would be a valuable endeavour to explore this fascinating enigma further as I postulate that FDCs may serve as reservoirs of both HIV and HHV-8 and may be a crucial cellular target that unlocks the secrets of HIV/HHV-8 associated CD.

It is also probable that some HHV-8 encoded viral products, homologous to cellular chemokines, induce inflammatory cytokine production in monocytes, macrophages, dendritic cells and endothelial cells which in turn influences HIV replication as MDDCs and T-helper cells are the most frequently HIV-infected cells in the bloodstream and primarily responsible for HIV dissemination in tissues and organs (Caselli et al., 2005).
We know that LANA-1 interacts synergistically with HIVtat protein in the transactivation of HIV LTR, which would therefore increase cell susceptibility to HIV in the presence of HHV-8. The alteration of DC function brought about by HHV-8 binding also affects T lymphocyte responses, which promotes an opportunistic environment for HIV to cause further deterioration of the immune system. In addition, in the presence of HHV-8, DCs were able to capture more HIV virus and increase transfer to susceptible cells (Liu et al., 2013). As there is increased susceptibility to HIV in both natural target cells such as T-lymphocytes and otherwise non-permissive cells, such as B-lymphocytes, this cumulatively increases the opportunity for B-lymphocytes to serve as reservoirs for both viruses.

The role of HIVtat in oncogenesis has been extensively studied. Studies show that HIVtat can also directly activate the lytic replication of HHV-8 by regulating the JAK/STAT signalling pathway (Aboul-ela et al., 1995). Other studies have demonstrated that tat also acts through additional pathogenetic mechanisms, which include upregulation of IL-6 expression and other angiogenic factors. Given the central role of IL-6 in the pathogenesis of MCD, HIV effects mediated by tat may also represent a central proliferative mechanism in MCD. Collectively, the above mentioned findings strongly suggest that tat plays a pivotal role in HHV-8-associated proliferations. The reverse mechanism of cooperation includes the activation of HIV LTR by herpesviruses that increases HIV replication, which further decreases immune competency and immune surveillance. HHV-8 takes advantage of this mechanism by also interacting physically and synergizing with HIVtat to increase retroviral transcription (Huang et al., 2001). Furthermore, tat increases the proliferative capacity and cell cycle progression of herpesvirus infected, immortalized B lymphocytes, which promotes B cell lymphomagenesis (da Silva & de Oliveira, 2011). It is therefore plausible that HHV-8 infected cells, in patients with concurrent HIV infection, likely come into contact with cells supporting active HHV-8 infection and replication, or even be co-infected by both viruses. Endothelial cells and monocytes may therefore represent other potential sites of interaction between these two viruses.
4.5.5 Dual staining LANA-1 and CD20

Immunohistochemical analysis of our cohort revealed occasional double-expressing LANA-1 and CD20 cells, correlated more with the findings of Dupin et al (1999), however Paravicini et al (1997) and Chadburn et al (2008) did not confirm these findings. Dupin et al (1999) also demonstrated that HHV-8 infected cells do not stain with immunoblast marker (CD79a) or B cell activation markers (CD23, CD38, CD30).

We already know that HHV-8 infection can expand the range of cells that could act as potential reservoirs for HIV infection by allowing for non-permissive B-cells to be become infected. Knowlton et al (2014) demonstrated that although HHV-8 targets naïve B-cells which include plasmablast-like cell populations, it is still unclear whether these B-cells are directly infected by the virus or if they are the result of infected precursors. Plasmablasts are rarely observed in normal lymph nodes.

Although HHV-8 infected B-cells comprise a very small proportion of the total cell population in CD, the plasmablastic subtype can show drastically increased numbers within the mantle zones. The plasmablasts in MCD are generally larger than the surrounding HHV-8 infected CD20 positive B-lymphocytes, harbour both latent (LANA-1) and lytic (vIL-6) profiles and do not express B-cell antigens (CD20, CD79a, PAX-5). They also express high levels of cytoplasmic IgM λ immunoglobulin, are usually MUM1+, CD138-, and CD30 weak+ (Dupin et al., 2000; Oksenhendler et al., 2002). Shulte and Talat (2010) went further in their immunohistochemical characterisation of these HHV-8 infected plasmablasts by successfully using double staining immunohistochemistry to demonstrate compartmentalization between plasmablasts and plasma cells. One consistent finding amongst all investigators was the downregulation of CD20 and other B-cell marker expression within the plasmablast population. This is important when we consider that rituximab therapy is usually successful.

Dupin et al (1999 and 2000) observed that at least 10-30% of the LANA-1 positive cells localized predominantly in the mantle zone of the follicles and that these plasmablasts were not present in HHV-8 negative MCD or HV-CD.

B-cell involvement was confirmed by studies that identified HHV-8 driven elaboration of both vIL-6 and hIL-6 by B-cells in the mantle zones of lymph nodes (Aoki et al., 1999;
Pinto and Nunes, 2011). In studies conducted by Chadburn et al. (2008) and Naresh et al. (2009), it was also observed that the majority of the HHV-8 infected cells within affected lymph nodes, were in fact B lymphocytes that supported either latency or lytic replication.

Our use of double immunohistochemical staining with CD20 and LANA-1 allowed us to identify virally infected B-cells as described by Naresh et al. (2009). Similar studies conducted on bone marrow biopsies of patients with HHV-8 associated MCD also found that a subset of LANA-1 positive cells were CD20 positive (Bacon et al., 2004). Our finding of LANA-1 and CD20 co-expressing cells, primarily extrafollicular in location, correlated with studies conducted by Chadburn et al. (2008), who characterised the infected cells as pre-terminally differentiated B cells. A study using *ex vivo* HHV-8 infection of tonsillar cells suggested that HHV-8 preferentially targets λ light chain-expressing B-cells for stable latent infection as it was demonstrated that there was a larger subset of λ than κ cells that were latently infected with HHV-8 (Hassman et al., 2011).

Our study allowed us to demonstrate that the number of the double expressing LANA-1/CD20 cells observed in the both the PC-CD and Pb-CD subtypes, although low, were still greater than numbers seen in the HV-CD subtype, findings which have not previously been reported. Low numbers of co-expressing cells are likely related to the downregulation of CD20 expression as a result of HHV-8 infection (Rappocciolo et al., 2008).

Another possible explanation is most likely due the fact that despite the significantly higher HHV-8 DNA levels in MCD, the virus persists mainly in its lytic form within B-lymphocytes (while remaining latent in KS- and PEL-infected cells) (Stebbing et al., 2011; Powles et al., 2009). Although B-cells are an important reservoir of latent HHV-8, other studies have also shown that other HHV-8 encoded lytic proteins, such as K8, K8.1 and vIL6 can also be detected in these infected cells, confirming that MCD is associated with lytic as well as latent HHV-8 infection (Dupin et al., 1999; Katano et al., 2000a).

It is therefore logical that some researchers have postulated that HHV-8 infection establishes both latent and lytic infection in naïve B-cell subsets, which most likely causes an early viral-driven cellular differentiation into plasmablasts in plasmablastic CD and a
pre-terminal plasma cell stage of differentiation in PC-CD, while some cells still continue to support the lytic cycle (Paravicini et al., 1997; Dupin et al., 1999).

4.5.6 Dual staining LANA-1 and CD3
Our study did not show LANA-1 and CD3 co-expressing cells which correlates with other authors who have previously demonstrated that infected cells do not stain with T-cell markers. (Paravicini et al., 1997; Dupin et al., 1999).

Specific MHC class I restricted (CD8+) responses to HHV-8 protein epitopes have been reported (Wilkinson et al., 2002). This highlights the important ability of cytotoxic CD8+ T lymphocytes (CTLs) to kill virally infected cells in response to intracellular viral antigen. These T-cell responses are possibly sufficient to control primary HHV-8 infection in immunocompetent individuals. DC-SIGN and DC-SIGNR are not expressed on T-cells and HHV-8 is not able to directly enter T cells (Geijtenbeek et al., 2000). In immunocomprised individuals however, it is also known that latent HHV-8 directly affects this ability of dendritic cells to activate virus specific CD8+ T cells (Rappocciolo et al., 2006). This most likely alters T cell responses and allows for active lytic and latent infection of susceptible cells, such as B-lymphocytes. Interestingly, CD4+ T-cells are suggested to support latent HHV-8 infection in B-cells by reducing the viral lytic cycle (Myoung & Ganem, 2011). This could possibly explain why even patients with adequate CD4 counts are susceptible to HHV-8 associated CD.

It has also previously been demonstrated that T-cell responses to HHV-8 antigens in HIV-infected individuals were low and that altered DC function by HHV-8 leads to decreased activation of T-cells (Guihot et al., 2006; Rappocciolo, et al., 2006). Based on previous epidemiological data, inadequate or altered T-cell function with ineffective control of virus replication, are important reasons for increased susceptibility to HHV-8 disease. This potentially implicates abnormalities that occur in DCs as a basis for T-cell dysfunction during HHV-8 infection.

4.6 HHV-8 negative cases
All the patients in our study fulfilled the histopathological criteria for CD, with 10 cases being immunohistochemically non-reactive for LANA-1 (5 HIV-positive and 5 HIV-negative).
A grading system has also been proposed for the pathological features observed in iMCD (0-3) and criteria for histopathological grading include germinal centre atrophy, follicular dendritic cell prominence, vascularity, germinal centre hyperplasia and plasmacytosis. It is interesting to report that our HHV-8 negative cases showed hypervascular pathology (atrophic germinal centres and FDC prominence) rather than plasmacytic features (hyperplastic germinal centres and plasmacytosis).

None of our HHV-8 negative cases (HIV-positive and HIV-negative) showed evidence of microscopic KS or the presence of plasmablasts. Although studies have shown that HIV infection itself is not necessary for KS development, it is associated with an increased incidence and a more aggressive course as HIV infected cells produce several inflammatory and growth promoting cytokines that influence the inflammatory milieu. The incidence of KS in our HIV seropositive patients compared with our HIV seronegative patients may be attributed to higher HHV-8 viral loads in those co-infected with HIV. One criterion not included in our study, but which would have added weight to the histopathological diagnosis, is detectable HHV-8 viraemia, either measured in the plasma or peripheral blood mononuclear cells.

More recently, Liu et al. (2016), in a systematic review, provided comprehensive information regarding the clinical features, treatments and outcomes of iMCD. Fajgenbaum et al. (2017) have presented an evidence based consensus of international major and minor diagnostic criteria development for the diagnosis of idiopathic MCD that is aimed at assisting in the prompt recognition and effective management of this disease.

4.7 Study limitations

As this was a retrospective study based solely on records kept in the Division of Anatomical Pathology, clinical data was limited. Data regarding viral load, CD4 counts and extent of disease were not available in some of our cases and additional information regarding whether patients were enrolled on HAART at time of diagnosis could not be retrieved. Retrospective studies are also subject to selection bias which can negatively impact the accuracy of the results. In addition, the audit was restricted to data obtained from a single hospital, specifically GSH. As GSH is a tertiary hospital, patients treated at this hospital are likely to have a greater burden of disease (i.e. lower CD4 counts, more
advanced HIV infection and greater seroprevalence of HHV-8) than those treated at local clinics. The prevalence and incidence rates of a disease are among the most fundamental measures in epidemiology, both of which differ across populations and time. Although our cohort number of 50 patients was the largest to date in South Africa, the number is still relatively small when compared to other cohorts in USA and the UK. It is imperative therefore that larger studies be conducted in South Africa so that important measures like prevalence and incidence estimates can be validated, the importance of which lies in establishing accurate disease burdens, susceptible populations at risk and survival outcomes across time and population groups.
CHAPTER 5:
CONCLUSION AND FUTURE RESEARCH

5.1 Conclusion
This study represented the largest cohort of HIV positive, HHV-8 associated Castleman disease patients in South Africa as well as the largest double-staining immunohistochemical analysis to date. Some of our results confirmed the findings of previous studies while other findings have not been described before and is our attempt to contribute to the expanding body of work already generated by this intriguing disease.

Our effective utilisation of LANA-1 in double staining immunohistochemical techniques not only identified co-staining DC-SIGN, DC-SIGNR and p24 cells, but increased numbers (statistically significant) in the plasmablastic subtype in comparison to other the CD subtypes which raises many additional questions regarding HIV-associated MCD. The plasmablastic CD subtype is morphologically characterised by ‘plasmablasts’ that show LANA-1 immunoreactivity. Pb-CD cases in our study showed an increased number of LANA-1 staining cells when compared to HV-CD (statistically significant) in addition to increased numbers of co-staining cells which may suggest that high HIV viral loads and/or low CD4 counts may be responsible for the findings observed.

Further to this, our findings of a statistically significant density of HHV-8 infected plasmablasts in Pb-CD, may potentially assist in identifying and/or refining specific histopathological criteria that could 1) predict HIV status in CD patient with an unknown HIV status, 2) predict outcomes in affected patients and 3) identify patients at risk of developing HHV-8 associated NHL (plasmablastic lymphomas or large B-cell lymphomas).

Our study also confirmed that DC SIGN and DC-SIGNR are both important requirements for HHV-8 entry into cells, that they represent a common ligand pathway utilized by both HIV and HHV-8 for viral entry and replication and that dendritic cells expressing DC-SIGN/DC-SIGNR are key players in HHV-8 infection and pathogenesis. I postulate, however, that dendritic cells that do not express DC-SIGN or DC-SIGNR such as follicular dendritic cells and Langerhans cells are also instrumental in dual HIV and HHV-8 replication and transmission.
We also confirmed that HHV-8 primarily infects cells that stain with CD20 and not cells that express common T-cell markers, such as CD3, which correlated with studies by previous investigators. It is still, however, unclear whether all B-cell lineage subsets are equally susceptible to HHV-8 lytic and latent infection, or if B-cell subset precursors are infected and as a result of active HHV-8 infection, driven into plasmablastic differentiation.

### 5.2 Future Research

I therefore feel that it is of fundamental importance to investigate if Pb-CD is directly associated with the severity of HIV-associated immunosuppression, which may ultimately reflect different pathogenetic mechanisms.

Future studies on larger cohorts of CD should also evaluate the correlation between the diagnosis of CD and the clinical state of immunosuppression as measured by the CD4 counts and HIV viral loads of the patient. Based on results from this study, it would also be interesting to see if any correlation exists between the degree of HHV-8 infection of B-cell subsets, specifically plasmablasts, and the CD4 count.

The prognostic implications of the CD4 counts at the time of diagnosis should at the same time also be evaluated through meticulous clinical follow-up of patients diagnosed with the disease. Should there be a correlation between the CD4 count and the degree of B-cell HHV-8 infection and eventually between the CD4 count and the prognosis of such a patient, it could serve as both histological and clinical prognostic markers for these patients. It is already known that the presence of HHV-8-positive plasmablasts in HIV-associated MCD identifies a distinct sub-group of patients with poor survival rates.

Although the potential for anti-DC-SIGN/DC-SIGNR therapeutic agents can be envisaged, there is still uncertainty whether these ligands are solely responsible for viral binding and entry. It has already been shown in previous studies that HHV-8 is capable of expanding the range of targets it infects as well as adapting to different host environments by utilising both ‘receptor flexibility’ and ‘molecular mimicry’. Full understanding of HHV-8 attachment biokinetics with different cellular targets is therefore imperative to the
development of targeted therapies that can prevent both primary infection and spread. Our results may also assist in providing further insight into the possible mechanisms that both HHV-8 and HIV utilise for immune evasion. The diverse and specialised functions of DC and its widespread distribution in the body undoubtedly plays an important contributory role in susceptibility of cells for both HIV and HHV-8 infection and therefore provides an important converging point for investigating the interaction between these two viruses.

The combined use of LANA-1 (HHV-8) and p24 (HIV) antibodies in our study, allowed us to not only identify co-infected cells, but to also observe their distribution within the lymph node, not previously described in other studies. Previous studies have shown that HHV-8 infects cells by binding to the DC-SIGN in a region that overlaps the HIV gp120 binding sites. Based on our results of co-infected cells, it may be possible to intentionally alter specific regions of DC-SIGN/DC-SIGNR that are key sites for binding of HHV-8 and HIV glycoproteins, which could potentially prevent viral infection. The similar binding sites in human DC-SIGN/DC-SIGNR for HHV-8 and HIV gp120 therefore represent novel targets for therapeutic interventions as well as microbicide and vaccine development efforts. The true relationship between HIV/AIDS, HHV-8 infection and CD should therefore be further considered in detailed molecular pathology, genetic and virology studies.

I also strongly believe that there may be broad genetic and molecular variation in CD which definitely warrants further investigation. The elucidation of the molecular pathways in this disease will not only assist in further refining its classification, but will also improve overall diagnosis.

Although there has been progress in the elucidation of many HHV-8 encoded proteins and their \textit{in vitro} functions, future studies investigating the role of HHV-8 infection and transformation of B-cells and endothelial cells in CD can assist in bridging the gap in our knowledge between the dendritic cell viral reservoir and lymphomagenesis in HHV-8 associated CD. There is also limited amount of data assessing T-cells and FDCs in MCD. FDCs may in fact play an important role in immune defense mechanisms against HHV-8 and FDC interactions with T- and B-cells therefore warrant further investigation.
Finally, I also propose that a grading system using defined histopathological and immunophenotypic criteria for each subtype of HHV-8+/HIV-associated CD, based on consensus haematopathological review, be established. This proposed grading system, while identifying important major and minor diagnostic criteria crucial for early and accurate diagnosis, should also include pertinent laboratory, histopathological and immunohistochemical findings.

It is our hope that the results of this study will not only generate further comprehensive understanding of the HIV/HHV-8 co-infection microenvironment that is crucial in the evolution of this disease, but will also potentially lead to improved therapeutic strategies for affected patients.
REFERENCES


Gherardi, R., Bélec, L., & Fromont, G. (1994). Elevated levels of interleukin-1 beta (IL-1 beta) and IL-6 in serum and increased production of IL-1 beta mRNA in lymph nodes of patients with polyneuropathy, organomegaly, endocrinopathy,M protein and skin changes (POEMS) syndrome. Blood, 83:2587–2593.


APPENDIX 1: DATA COLLECTION TEMPLATE

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<th>Case No.</th>
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APPENDIX 2: ETHICS PROTOCOL APPROVAL

FHS017: Annual Program Report / Renewal
Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

HREC office use only (FWA00001837; IRB00001938)
This serves as notification of annual approval, including any documentation described below.

☑ Approved Annual progress report Approved until/next renewal date 20.5.2018
☐ Not approved See attached comments

Signature Chairperson of the HREC Date Signed 05/05/2018

Principal Investigator to complete the following:

1. Protocol Information
date (when submitting this form) 05 May 2017
HREC REF Number 14-02012 Current Ethics Approval was granted until 30.05.2017
Protocol title Interaction between SC Sign SC Sign + HIV Cytokines in GVHD
Principal Investigator Dhoshinee Rama Chetty
Department / Office Internal Mail Address Anatomical Pathology - NHLS - D3 Groote Schuur Hospital
1.1 Does this protocol receive US Federal funding?
☐ Yes ☐ No

2. Protocol status (tick ✔)
☐ Research-related activities are ongoing
☒ Data collection is complete, data analysis only

Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository.

3: Protocol summary
Total number of records or specimens collected, reviewed or stored since the original approval 50
Total number of records or specimens collected, reviewed or stored since last progress report 50
Have any research-related outputs (e.g. publications, abstracts, conference presentations) resulted from this research? If yes, please list and attach with this report.
☐ Yes ☐ No

4. Signature
Signature of PI Date 05 May 2017

23 July 2014 Page 1 of 1
(Note: Please complete the Closure form (FHS019) if the study is completed within the approval period)