

The Pathogenesis of HIV-associated cardiomyopathy: a histological, virological, and genetic study

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MSc (Paed)

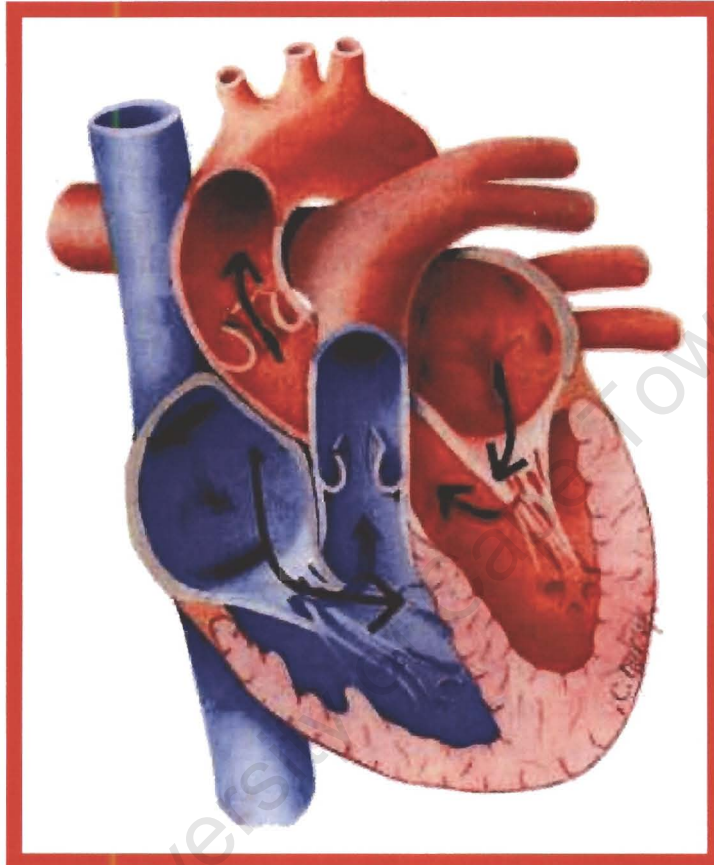
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In the Division of Human Genetics
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The heart



*"The future belongs to those who believe in
the beauty of their dreams."*

Eleanor Roosevelt

Declaration

This study was performed between 2003 and 2007 under the supervision of Professor Bongani M. Mayosi from the Department of Medicine at the University of Cape Town.

I hereby certify that this thesis is my own work and has not been presented for a degree at any other university.

Gasnat Shaboodien

Signed by candidate

February 2008

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ABSTRACT

This study was designed to elucidate the pathogenesis of HIV-associated cardiomyopathy by means of histological, virological, and genetic methods. We hypothesised that HIV-associated cardiomyopathy was due to myocarditis in a sub-set of people who were genetically predisposed to develop heart muscle disease when infected with cardiotropic viruses. The objectives were to (1) define the histological abnormality in HIV-associated cardiomyopathy using light microscopy, immunohistochemistry, and electron microscopy, (2) to establish whether cardiotropic viruses played a role in the pathogenesis of HIV-associated cardiomyopathy, and (3) to determine if the mitochondrial T16189C variant with a poly-C tract (i.e., 'OriB' variant) predisposed to cardiomyopathy in people who are infected with HIV.

To address these objectives, an endomyocardial biopsy study was conducted in which the histological, virological and genetic changes in patients with HIV-associated cardiomyopathy were compared with findings in patients with idiopathic dilated cardiomyopathy and heart transplant recipients.

The histological analysis showed that active/acute myocarditis accounted for a minority of patients with HIV-associated cardiomyopathy (approximately 25%) in Cape Town, with the remainder exhibiting borderline/chronic myocarditis (~50%) or no evidence of myocardial inflammation (~25%). This is in contrast to studies elsewhere which have suggested that active/acute myocarditis is the pathological hallmark of HIV-associated cardiomyopathy. The virological analysis revealed that cardiac infection with Epstein-Barr virus, Herpes Simplex virus, enterovirus and adenovirus to be common in patients with HIV-associated cardiomyopathy and heart transplant recipients whilst parvovirus and cytomegalovirus infection was uncommon. Enteroviruses

dominated the tissue in the idiopathic dilated cardiomyopathy cohort. Light and electron micrographs also revealed nuclear abnormalities similar to those seen in inherited cardiomyopathies. In the genetic analysis, the mitochondrial OriB variant was not associated with an increased risk of HIV-associated cardiomyopathy. Furthermore, a large replication study involving 99 cases of idiopathic dilated cardiomyopathy and 117 normal controls failed to confirm the original proposition that the OriB variant is a genetic risk factor for idiopathic dilated cardiomyopathy in Africans. A meta-analysis indicated that the OriB variant did however predispose to the development of idiopathic dilated cardiomyopathy in the Caucasian population (OR 2.15; 95% CI 1.16-3.98; P=0.01).

We show, in this first molecular pathological and genetic study of its kind, that HIV-associated cardiomyopathy encompasses the full spectrum of inflammatory cardiomyopathy.

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Alghamdoelielah

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LIST OF ABBREVIATIONS

AIDS-acquired immune deficiency syndrome
Adv2, 5- adenovirus 2,5
AZT- zidovudine
CAR receptor-coxsackie-adenovirus receptor
CVB3-coxsackie virus B3
DAF receptor- decay accelerator factor
DNA-deoxyribonucleic acid
EBV- Epstein-Barr virus
HAART-highly active antiretroviral therapy
HIVAC -HIV-associated cardiomyopathy
HTx-heart transplant recipient
HIV-human immunodeficient virus
HHV6-human herpesvirus 6
HSV-Human Simplex virus
iDCM- idiopathic dilated cardiomyopathy
IHC-immunohistochemistry
MHC I- major histocompatibility complex I
MHCII- major histocompatibility complex II
PCR-polymerase chain reaction
PVB13- parvovirus B13
RNA-ribonucleic acid
RT-PCR- reverse transcriptase polymerase chain reaction
Tat-transactivator protein

CHAPTER 1

INTRODUCTION

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CHAPTER 1: INTRODUCTION

1.1 EPIDEMIOLOGY OF HIV-ASSOCIATED CARDIOMYOPATHY

Early in the acquired immune deficiency syndrome (AIDS) epidemic a lot of attention was paid to systemic organ failure (in the pulmonary, gastrointestinal, renal and central nervous systems) as a result of the global immune paresis caused by human immunodeficiency virus (HIV) infection. Clinical cardiac involvement however, received little attention except as a site of spread of Kaposi sarcoma. The discovery of the development of fatal cardiac tamponade in patients with AIDS prompted Fink to examine by echocardiography a series of patients with AIDS in order to determine the prevalence of cardiac abnormalities (Fink 1984). This led to the finding that 73% of individuals with AIDS had cardiac abnormalities on echocardiography, which were usually subclinical. The era of HIV associated cardiac disease had arrived.

HIV associated cardiomyopathy is characterised by global systolic functional impairment with or without left ventricular dilation (Magula and Mayosi 2003). While some patients with left ventricular dysfunction present with features of cardiac failure, the majority has left ventricular dysfunction, detected only by echocardiography, without any clinical suggestion of heart failure. The importance of cardiac dysfunction in people living with HIV is demonstrated by its severe negative effect on survival in patients who are not treated with anti-retroviral drugs; the median survival to AIDS-related death is 101 days in patients with left ventricular dysfunction compared to 472 days in patients with echocardiographically normal hearts at a similar stage of infection (Currie 1994).

Although pathologic studies and case reports of HIV patients with dilated cardiomyopathy, pericardial effusion, Kaposi sarcoma and opportunistic infections involving the heart were described early in the

history of the AIDS epidemic (Cohen 1986, Anderson 1988, Lafont 1988, Fink 1984), the prevalence of cardiac abnormalities in adults living with HIV infections remained unknown until the late 1980s (Himelman 1989). To better define the prevalence, Himelman and others used two-dimensional echocardiography with Doppler ultrasound studies on a series of consecutive patients with HIV infection. They went on to report that patients with HIV-associated dilated cardiomyopathy had a poor prognosis, with 50% of subjects dying within six months (Himelman 1989, Factor 1989, Currie 1994, Feeley 2000).

The annual incidence is reported to be 15.9 per 1 000 asymptomatic HIV-positive patients who are not on highly active antiretroviral therapy (HAART), and prospective natural history studies indicated that about 8% of people living with the HIV develop cardiomyopathy in their lifetime (Barbaro 2002). In Africa, a continent with the largest number of people living with HIV in the world (Figure 1), cross-sectional and retrospective studies suggest that left ventricular dysfunction is a common cardiac abnormality in acutely ill HIV patients who are admitted to hospital (Magula and Mayosi 2003, Ntsekhe and Hakim 2005). Studies of outpatient and inpatient HIV-infected cases suggest a prevalence of cardiomyopathy of 9-57% (Ntsekhe and Hakim 2005). The risk factors for the development of cardiomyopathy in HIV-infected African patients who are not receiving anti-retroviral therapy are low-socio-economic status, advanced HIV disease, and low levels of plasma selenium (Twagirimukiza 2007).

Currently, a large spectrum of HIV-associated cardiovascular abnormalities including pericarditis, cardiomyopathy, pulmonary hypertension, vascular abnormalities, and drug-associated coronary artery disease (Ntsekhe and Hakim 2005) are described. Most studies indicate that the HIV-associated cardiac diseases of major clinical importance are cardiomyopathy and pericardial disease (Magula and Mayosi 2003). A systematic review on the spectrum of cardiac

involvement in HIV-infected people living in Africa has revealed a paucity of studies on the pathogenesis of HIV-associated cardiomyopathy on the continent (Magula and Mayosi 2003), which is the subject of this work.

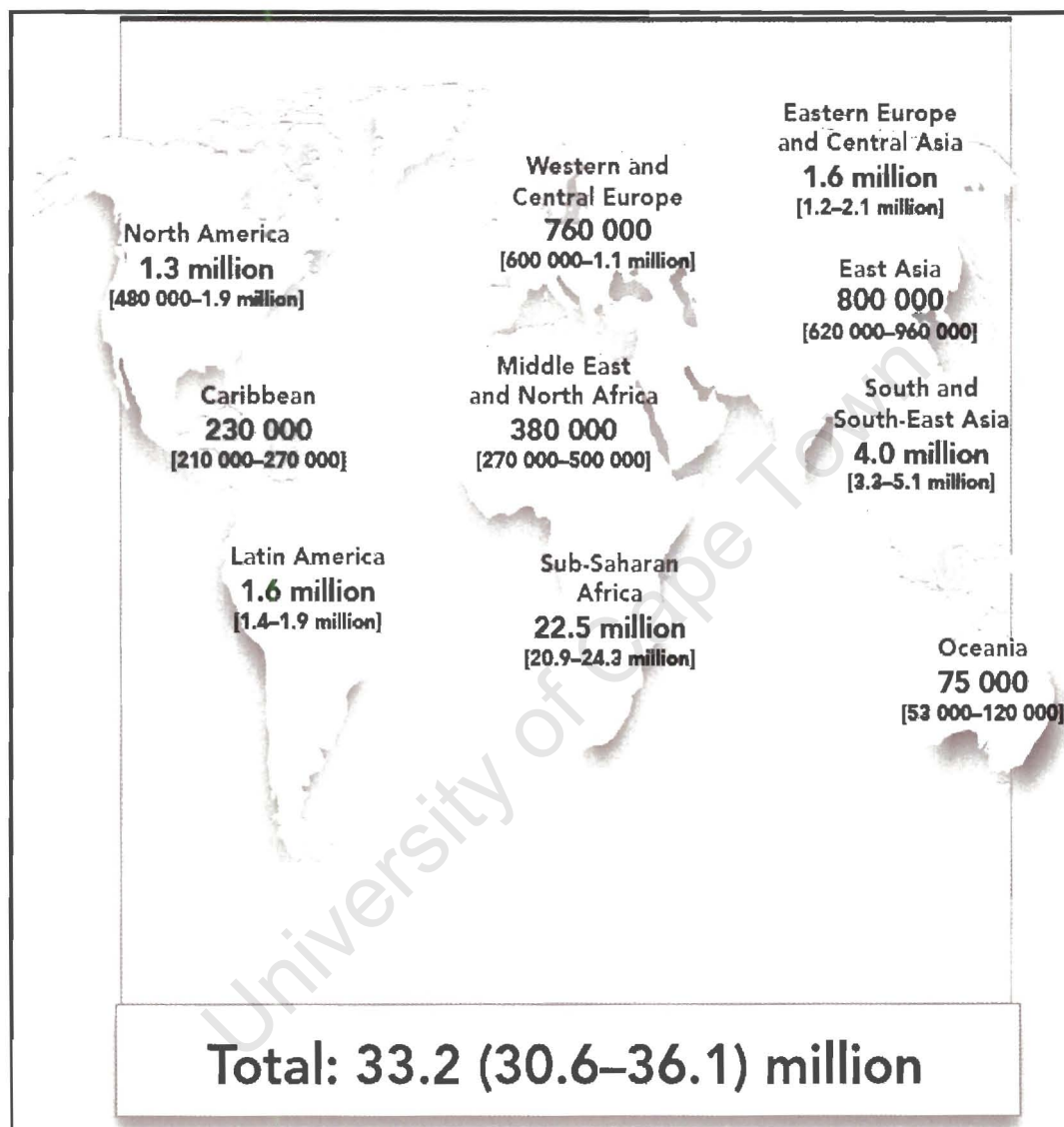


Figure 1 Summary of global estimates of adults and children living with HIV in 2007 (URL 1).

1.2 PATHOGENESIS OF HIV-ASSOCIATED CARDIOMYOPATHY

The precise cause of HIV-associated cardiomyopathy remains unclear (Currie and Boon 2003). However, a number of hypotheses regarding the pathogenesis of the condition have been proposed, including (1) myocarditis due to direct infection with HIV or other cardiotropic agents, (2) genetic predisposition, (3) post-infective cardiac autoimmunity, (4) nutritional deficiencies, and (5) cardiotoxic effects of drugs.

1.2.1 MYOCARDITIS

An association between cardiac dysfunction and myocarditis reported in AIDS necropsy series appears to be probable, and is analogous to the current thinking on the pathogenesis of idiopathic dilated cardiomyopathy (Currie and Boon 2003). Although serious cardiac abnormalities have been found to be more common in HIV-infected patients with definite myocarditis than in those with normal histology, there often appears to be only a weak correlation between left ventricular dysfunction and histological evidence of myocarditis (Reilly 1988, Baroldi 1988, Anderson 1988). Therefore, an unequivocal connection between myocarditis and cardiomyopathy in AIDS patients has yet to be wholly confirmed (Currie and Boon 2003) .

The histological diagnosis of acute or definite myocarditis is based on the identification of inflammatory cells infiltrating the myocardium with destruction of myocytes (Magnani 2006). The term 'borderline myocarditis' is used in situations where an inflammatory infiltrate is found in isolation. Controversy has, however, surrounded the diagnosis of myocarditis, with its wide variety of etiologies, its heterogeneous clinical presentations, and the lack of uniformity in diagnostic classification. The methodologies used in previous studies (light microscopy, viral culture, Western blot, and serology) appeared to definitively identify only a portion of patients with myocarditis; additional

methods were needed to improve the diagnostic accuracy in cases of suspected myocarditis. Two diagnostic classification systems, the Dallas (Aretz 1987) and Marburg criteria, were established to aid in the diagnosis of myocarditis. Differences in the techniques used to diagnose myocarditis, compounded by possible sampling errors, make the prevalence of myocarditis in HIV populations difficult to establish, with estimates ranging from 12.5% to 53% (Currie and Boon 2003).

1.2.1.1 Primary HIV myocarditis

It has been postulated that HIV associated cardiomyopathy may be due to the direct infection of myocardial cells by HIV (Barbaro 1998). Although it is clear that interstitial cells within the myocardium may be infected with HIV (Wu 1992, Lipschultz 1998), there is only limited evidence to suggest that the virus can enter the cardiac myocyte (Grody 1990). Contamination with the patient's own infected blood cells can make interpretation of polymerase chain reaction (PCR) and cell culture investigations difficult. Immunohistochemical studies have shown no evidence of gp 120 or p24 antigen expression in myocytes (Flomenbaum 1989, Jacob 1992).

Early *in situ* hybridization (ISH) studies identified HIV signals from myocytes (Grody 1990, Lipschultz 1990, Herskowitz 1993), but the HIV hybridization signal did not correlate well with histological evidence of myocarditis as most of the positive signals appeared in relatively normal myocardium. Precise identification of cells targeted by ISH is difficult as they are often obscured by the positive reaction that occurs as part of the technique. Other *in situ* hybridization studies though, have failed to demonstrate HIV within the myocardium (Dittrich 1988) despite microscopic evidence of both definite and borderline myocarditis (Beschorner 1990, Wu 1992).

In a study by Rodrigues (1991), PCR demonstrated the presence of the HIV sequences in myocytes but HIV antigens were also found in

dendritic cells in all patients with cardiac symptoms and 60% of the patients without symptoms. Although their results did not support a direct role of the HIV in myocardial dysfunction, the results suggested that the interstitial dendritic cells may be involved in the development of the cardiac dysfunction observed in HIV infected patients (Rodrigues 1991).

Although *in vitro* studies have shown that HIV is unable to enter skeletal myocytes, others have shown that HIV can enter cells that do not have CD4 receptors (Ikeuchi 1990, Herskowitz 1992). It is possible, therefore, that infiltrating lymphocytes, endothelial cells (Dittrich 1988), macrophages or other antigen presenting cells may facilitate HIV entry into CD4 negative host cells (Robelledo 1998).

Therefore, it still remains possible that HIV could play a direct role in the pathogenesis of HIV-related heart muscle disease.

1.2.1.2 Secondary HIV myocarditis

1.2.1.2.1 Cardiotropic viruses

Literature has shown enteroviruses, adenoviruses and parvovirus to be the common cardiotropic viruses for the pathogenesis of human viral myocarditis and the development to inflammatory dilated cardiomyopathy in patients who survive the initial infection (Griffin *et al* 1995). Key interaction sites of viruses with their hosts are depicted in Figure 2.

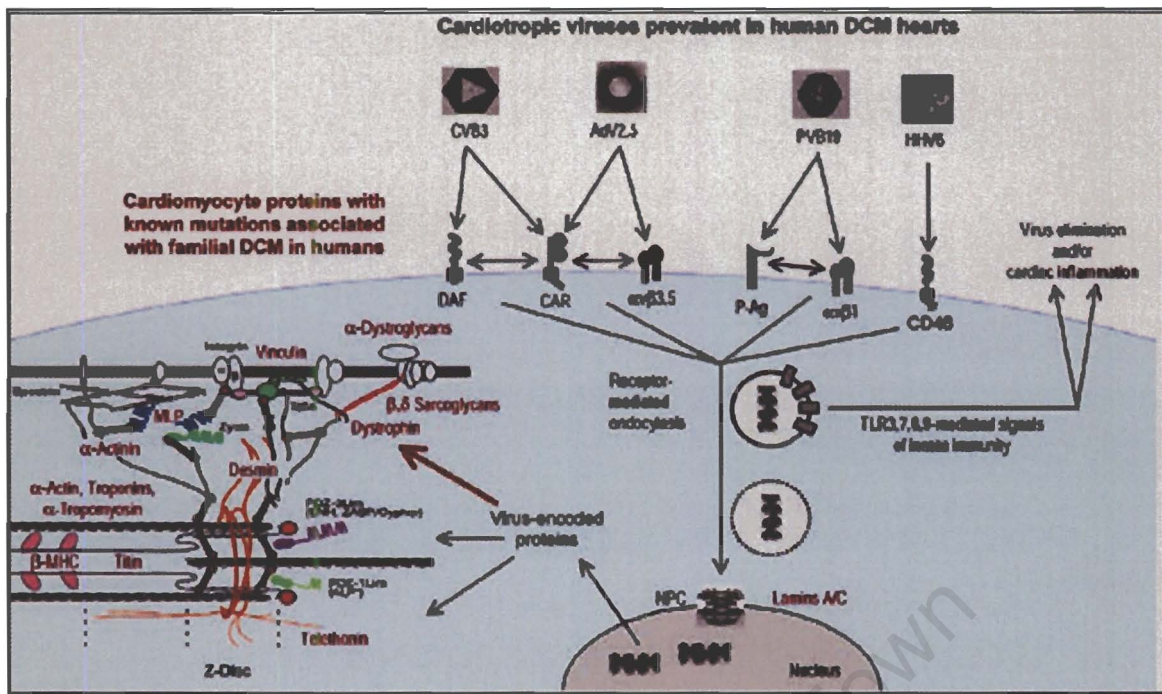


Figure 2 Cellular entry mechanisms for most cardiotropic viruses via cell surface receptors and interacting co-receptors, followed by receptor-mediated endocytosis of the virus particles, release of the viral RNA (CVB3) or DNA genomes (other viruses), and their nuclear import via the nuclear pore complex (NPC). CVB3-coxsackie virus B3; PVB13-parvovirus B13; AdV- adenovirus; CAR-coxsackievirus-adenovirus receptor; DAF-decay accelerating factor; HHV6; human herpes virus 6 (Poller *et al* 2005).

With the introduction of the nucleic acid recombination technologies it became possible to assess the presence of viral genomes in myocardial tissue of patients with idiopathic dilated cardiomyopathy or myocarditis (Giacca 1994). The initial report by Bowles showed that enteroviruses were capable of persisting in the myocardium beyond the acute phase of myocarditis, thus suggesting a role in the aetiology of idiopathic dilated cardiomyopathy, supporting the hypothesis that enterovirus-induced myocarditis predisposes to the development of idiopathic dilated cardiomyopathy (Bowles 1986). After this report more investigators used molecular biology techniques to study the aetiology of

viral myocarditis, and in particular, the role of enterovirus persistence in the progression of acute myocarditis to chronic myocarditis and even idiopathic dilated cardiomyopathy (Bowles 1989, Archard 1991, Grasso 1992).

Because of its sensitivity and specificity for detecting nucleic acid sequences in cardiac tissue, PCR became the 'gold standard' for viral detection, but divergent PCR findings have been reported regarding the presence of infectious agents in the hearts of patients with idiopathic dilated cardiomyopathy (Jin 1990, Keeling 1992, Kishimoto 1994).

Other researchers went on to report that, although enteroviruses were an important causative agent of myocarditis and idiopathic dilated cardiomyopathy, adenovirus infection was also important in the pathogenesis of left ventricular failure in adults (Noutsias 2003, Kuhl 2003, Bowles 2003). Pauschinger (1999) reported that their histologic analysis demonstrated that adenovirus-positive patients typically had mild or borderline myocarditis by the "Dallas" criteria, whereas enteroviruses were more commonly associated with acute myocarditis as well as the presence of inflammatory infiltrates in the idiopathic dilated cardiomyopathy patients (Pauschinger 1999). Bowles suggested that infection of the myocardium with adenovirus may result in less immune cell activation than with other agents, suggesting the possibility of a different mechanism of pathogenesis of adenovirus-induced chronic myocardial disease (Bowles 1999, Bowles 2003).

Studies on myocarditis or idiopathic dilated cardiomyopathy patients found viral prevalence rates to range from 20-71% in endomyocardial tissues to 21-40% in autopsy tissues (Martin 1994, Griffin 1995, Baboonian and Treasure 1997, Barbaro 1998, Pauschinger 1999, Hogrefe 2002, Bowles 1999, Bowles 2003, Kuhl 2003, Kuhl 2005, Poller 2005). Using PCR, Bowles and others found a 31-42% frequency of viral genomes in HIV associated cardiomyopathy patients

(Bowles 1999, Barbaro 1998, Barbaro and Lipschultz 2001) with and without an inflammatory infiltrate. In a study by Barbaro (1998) they reported 35-52% detection of HIV-1 nucleic acids in HIV associated cardiomyopathy patients; 86% of these patients had active myocarditis and 32% were co-infected with coxsackie virus. *In situ* hybridization revealed a high frequency of either cytomegalovirus (CMV) or HIV or both in HIV-associated cardiomyopathy patients (Barbaro 1998, D'Amanti 2001, Barbaro 2001, Barbaro 2005).

Herskowitz detected myocarditis due to CMV in 48% of HIV-associated cardiomyopathy patients (Herskowitz 1994). This report was confirmed by Bowles when they reported the 42% prevalence of CMV/adenovirus in HIV-associated cardiomyopathy myocardial tissue (Bowles 1999). Currie and Boon summarized these data by reporting that even though CMV infection is known to infect immunocompromised individuals and is commonly found in AIDS patients, CMV has only infrequently been found as the cause of myocarditis in HIV positive patients (Currie and Boon 2003).

1.2.1.2.2 Non-viral opportunistic infections

A range of studies have identified a diverse assortment of non-viral opportunistic pathogens in the myocardium of patients with HIV associated cardiomyopathy, including *Toxoplasma gondii*, *Cryptococcus neoformans*, *Pneumocystis carinii*, *Candida albicans*, *Mycobacterium tuberculosis* and *Histoplasma capsulatum*, but there was no clear correlation between the presence of an opportunistic pathogen and the severity of myocarditis (Lafont 1988, Himelman 1989, Anderson 1988). Non-viral opportunistic infectious diseases were not a common cause of myocarditis in the pre-HAART era in the developed world, where cardiotropic viruses were implicated in the majority of cases. In Africa, a small series of 16 patients with AIDS were studied postmortem. Non-

viral opportunistic infections such as *Toxoplasma gondii* was found in 18.75%, *Cryptococcus neoformans* in another 18.75% and *Mycobacterium avium intracellulare* in 12.5% (Longo-Mbenza 1998).

1.2.1.2.3 Viral accessory protein-mediated damage

HIV-associated cardiomyopathy may also result from the toxic effects of HIV accessory proteins, a mechanism of damage that underlies the neuropathogenesis of HIV dementia (Nath 2002). It is likely that reservoir cells (such as dendritic cells) in the myocardium and the cerebral cortex, which are not susceptible to treatment, may harbor HIV and may chronically release toxic viral proteins, contributing to progressive and late damage in both systems. This infection of dendritic cells by HIV explains one mechanism by which the virus could persist after prolonged HAART.

While there is ample evidence to support the role of virotoxins in HIV dementia (Nath 2002), there has been very little work done to examine the role of HIV accessory proteins in HIV-associated cardiomyopathy (Twu 2002). There is a growing body of literature that suggests that the HIV accessory proteins Nef and Vpr could be involved in the depletion of CD4+ and non-CD4+ cells and tissue atrophy, and in delaying the death of HIV-infected cells. Cell depletion is likely to be predominantly a bystander effect because the number of cells dying far outnumbers HIV-infected cells and is not confined to CD4(+) cells. The myristylated N-terminal region of Nef has severe membrane disordering properties, and when present in the extracellular medium causes rapid lysis *in vitro* of a wide range of CD4(+) and non-CD4(+) cells, suggesting a role for extracellular Nef in the depletion of bystander cells (Azad 2000).

A mouse model has been reported in which targeted myocardial transgenic expression of the HIV transactivator (Tat) protein caused

cardiomyopathy and mitochondrial damage (Raidel 2002). HIV Tat serves as a transactivator that stimulates transcription and is required for efficient HIV replication. In addition, Tat is known to cause oxidative damage in organs and has been shown to decrease the glutathione content in livers of transgenic mice. Selective expression of Tat in the myocardium of transgenic mice increased left ventricular mass, decreased ventricular fractional shortening, caused mitochondrial destruction, and resulted in selective depletion of cardiac glutathione. These data suggest that myocardial expression of Tat could contribute to HIV cardiomyopathy in man if its expression could be conclusively proved in wild type HIV infection.

1.2.1.2.4 Cytokine-mediated damage

It is also possible that myocardial dendritic cells play a key role in the interaction between HIV, the myocyte and the activation of cytotoxic cytokines (Rodriguez 1991, Barbaro 1999). In HIV disease, dendritic cells can initiate the primary immunologic response and present the antigen to T-lymphocytes. The interaction between dendritic cells and T lymphocytes, particularly CD8 cells, could promote a local elevation in the multifunctional cytokine tumor necrosis factor (TNF)- α , which can also be produced and secreted by infected macrophages (Luster 1998). TNF- α produces a negative inotropic effect by altering intracellular calcium homeostasis, possibly by inducing nitric oxide (NO) synthesis, which also reduces myocyte contractility (Finkel 1992, Yokohama 1992). Thus the HIV myocardial infection, the interaction between HIV and cardiotropic viruses, and the immunodeficiency may all enhance the inflammatory response and increase both the expression and the cytotoxic activity of specific cytokines, such as TNF- α and inducible NOS and cause progressive tissue damage by chronic release of cytotoxic

cytokines (Schrager 1998, Freeman 1998, Barbaro and Lipschultz 2001).

1.2.2 GENETIC PREDISPOSITION

HIV disease is characterized by extreme heterogeneity in clinical course, which is probably a result of host genetic differences and variability in virulence factors among HIV strains (Currie 1994). It is of great interest that only a subset of people who are infected with HIV develop heart muscle disease, which may be a result of an interaction between host and viral genetic factors and the environment. Studies of patients with idiopathic dilated cardiomyopathy, a disease that often follows unrecognized viral myocarditis, have shown that about 30% of cases may be due to genetic factors (Michels 1992; Keeling 1992). During the past few years some progress has been made in the identification of genetic susceptibility factors to idiopathic dilated cardiomyopathy (Komajda 2000). Furthermore, a number of host genetic variants have already been identified to be associated with HIV disease progression, and to a much lesser extent with resistance to infection (Carrington 2001).

There is an emerging body of literature that has identified a number of genetic factors that predispose to cardiomyopathy in Africans in the general population. An association with human leukocyte antigen (HLA)-DR1 and DRw10 antigens has been reported in South African patients, implying that genetically-determined immune-response factors play a role in the pathogenesis of dilated cardiomyopathy in a subset of individuals (Maharaj 1990). A common mitochondrial DNA polymorphism (T16189C, also known as the ORiB variant) has also been found to be a genetic risk factor for dilated cardiomyopathy in a South African cohort, with a population attributable risk of 6% (Khogali 2001). It is not known,

however, whether these or other specific genetic factors play a role in the development of HIV-associated cardiomyopathy (Lipshultz 1998).

1.2.3 POST-INFECTIVE AUTOIMMUNITY

It has been suggested that HIV infection may trigger a diverse range of autoimmune phenomena in patients who are predisposed to autoimmune disease by HLA or non-HLA genes. Hypergammaglobulinaemia, elevated circulating immune complexes, and autoantibodies are well described in HIV-infected individuals (Golding 1988, Klaassen 1991).

The combination of viral hybridization and increased myocardial expression of MHC class I strongly suggests an active immune process in the myocardium (Barbaro 1998). Herskowitz detected IgG anti-myosin antibodies in four of six AIDS patients with cardiomyopathy; these antibodies were not detected in HIV-positive patients without cardiomyopathy (Herskowitz 1993, Herskowitz 1994). Others supported the theory that cardiac autoimmunity play a role in the pathogenesis of HIV-related heart disease and suggested that cardiac autoantibodies may be markers of left ventricular dysfunction in HIV-positive patients with previously normal echocardiographic findings (Currie 1998, Meldrum 1998, Currie and Boon 2003).

Superantigens also play an important role in the pathogenesis of many diseases by forming a trimolecular complex with MHC class II molecules on the antigen-presenting cells on the T lymphocyte receptor (Johnson 1998). The binding results in a massive stimulation of T lymphocytes. In HIV infection, the HIV regulatory protein (*Nef*) binds to MHC class II on antigen-presenting cells, and the T lymphocytes become activated. This stimulates the release of cytokines such $\text{TNF-}\alpha$, interferon- γ , and interleukin (IL)-2. Proliferation of B cells may result in hypergammaglobulinemia. An autoimmune response may occur as a

result of B cell differentiation into immunoglobulin-secreting cells and the activation of T lymphocytes (Barbaro and Lipschultz 2001)

Common viruses could also play a role in causing or facilitating the onset of cardiac autoimmunity in HIV infected patients. For example, cardiotropic viruses could modify existing myocyte surface antigens or expose other hidden cell surface epitopes, causing an aberrant autoimmune response against endogenous autoantigenic peptides associated with the MHC molecules (Herskowitz and Ahmed-Ansari 1992). This myocyte antigen presentation could be responsible for the ongoing inflammation and cardiac injury seen in many cases of myocarditis, although the precise mechanism behind this remains poorly understood. Experimental evidence for these theories has been found in studies on cytomegalovirus myocarditis in mice (Lodge 1987, Price 1991, Currie and Boon 2003).

In fact, both cell-mediated and humoral immunity have been postulated to have an important pathogenic role in the development and progression of the viral cardiomyopathic process itself (Barbaro and Lipschultz 2001).

1.2.4 NUTRITIONAL DEFICIENCIES

Nutritional deficiency is common in HIV infection and, in particular, abnormally low levels of selenium and antioxidant vitamins have been demonstrated (Kavanaugh-McHugh 1991, Twagirumukiza 2007). It has been postulated that 'oxidative stress' may be an important mechanism for cellular damage in AIDS (Fuchs 1991). Selenium deficiency is implicated in the pathogenesis of Keshan disease, a form of dilated cardiomyopathy seen in China. Keshan disease may be prevented by widespread dietary supplementation and, likewise, abnormal left ventricular dysfunction was seen to resolve in a small group of HIV-

infected patients after treatment with a selenium salt (Keshan Disease Research Group 1979, Zazzo 1998, Currie and Boon 2003).

Patients infected with HIV are predisposed to a poor nutritional status due to a combination of factors including inadequate dietary intake, intercurrent illness, and malabsorption (Hoffman 1999). Malnutrition and wasting are independent predictors of cardiac-related morbidity and mortality in children living with HIV (Luginbuhl 1993). Micronutrient deficiencies as a result of protein energy malnutrition are also common. Decreased levels of selenium, carnitine and thiamine have been reported in HIV positive patients (Hoffman 1999).

The pathogenic role of selenium, a potent antioxidant that is an important cofactor for immune function (Rayman 2000), is of particular interest in patients with HIV infection and heart muscle disease. *In vivo* studies have shown that selenium plays an important role in protecting cells from HIV activation (Sappey 1994). Patients with HIV infection are prone to develop selenium deficiency due to the trapping of selenium by a selenoprotein encoded by the proviral genome (Chariot 1999). Selenium deficiency may allow the proliferation of HIV (Sappey 1994), and enhance the virulence of coxsackie virus to cardiac tissue (Beck 1998). A small case series, involving a total of less than 10 patients, have reported on the association of selenium deficiency with cardiac dysfunction in people living with HIV (Kavanaugh-McHugh 1991; Chariot 1999). However, the role of this potentially reversible cause of cardiomyopathy remains to be confirmed in larger studies. The identification of selenium deficiency as a determinant of cardiomyopathy could have major implications for the treatment of patients with heart failure.

1.2.5 CARDIOTOXIC EFFECTS OF DRUGS

Zidovudine (AZT) acts as a competitive inhibitor of HIV reverse transcriptase but also inhibits mitochondrial DNA polymerase (Simpson

1989). This can cause a specific, dose-dependent, reversible skeletal myopathy (Peters 1993) in which specific histological changes have been noted, including focal necrosis, cytoplasmic bodies, mitochondrial abnormalities, and ragged, red fibres in the sarcoplasm (Fischl 1989). These are similar to those discovered in the myocardium of rats fed with the drug (Dalakas 1990, Currie and Boon 2003). One study suggests that AZT is associated with diffuse destruction of cardiac mitochondrial ultrastructure and inhibition of mitochondrial DNA replication (Lewis 1994).

The role of AZT in the pathogenesis of HIV related heart muscle disease in humans remains unclear. In a study of 26 HIV-infected patients with cardiac failure, 13 of whom had been treated with AZT, three individuals developed cardiac dysfunction during treatment, which resolved following its discontinuation (Lipschultz 1992). However, AZT did not appear to ameliorate or worsen the cardiac abnormalities in a prospective study of 24 children (Lipschultz 1992) and, in earlier adult studies, the drug did not appear to be an important factor for the development of cardiac dysfunction (Jacob 1992, Currie and Boon 2003).

Other antiretroviral drugs, such as didanosine and zalcitabine, do not seem either to promote or to prevent dilated cardiomyopathy. Highly active antiretroviral therapy (HAART) regimens, which combine two nucleoside-transcriptase inhibitors and one protease inhibitor (PI), have been associated with an increased risk of cardiovascular complications (Rerkpattanapipa 2000, Sorelle 1998). The metabolic alterations induced by protease inhibitors may enhance the pathogenic action of pre-existing cardiovascular risk factors with an increase of acute coronary syndromes, even in young individuals, generally related to the duration of protease inhibitor treatment and the type of protease inhibitor used (Sullivan 1998, Henry 1998, Jutte 1999, Flynn and Bricker 1999).

There is good evidence that HAART reduces the incidence of cardiovascular manifestations of HIV infection (Pugliese 2000). One Italian study reported the prevalence at 1.8%, an almost 7-fold reduction from the pre-HAART era. But in Africa, only 5% of infected adults who need HAART receive it. African countries are only able to spend 1000 times less on care per HIV infected patient than the United States. There is a glimmer of hope though, with several large international funding initiatives having been launched to tackle the HIV/AIDS epidemic in Africa (Ntsekhe and Hakim 2005).

1.3 HYPOTHESIS

The pathogenesis of HIV-related heart muscle disease is undoubtedly complex, and is most probably multifactorial (Currie and Boon 2003). There is a paucity of studies that have examined the cause of cardiomyopathy in HIV-infected people living in Africa (Magula and Mayosi 2003). To the best of my knowledge, there is one report of cardiac histology in 16 HIV-positive patients with cardiomyopathy who were examined post mortem (Longo-Mbenza 1998). All 16 patients with HIV-associated cardiomyopathy had histopathological evidence of acute myocarditis, which was attributed to *Toxoplasma gondii* (3/16), *Cryptococcus neoformans* (3/16), *Mycobacterium avium intracellulare* (2/16) and to HIV in the rest of the series (8/16). The results of this small study raise the prospect that HIV-associated cardiomyopathy may be due to potentially treatable opportunistic infections in up to 50% of cases. By contrast, in Western series, opportunistic viral infections have been implicated in a significant proportion of cases.

To the best of my knowledge, we have conducted the first endomyocardial biopsy study of the pathogenesis of HIV-associated cardiomyopathy in patients living in Africa. We hypothesized that HIV-associated cardiomyopathy is due to myocarditis in individuals with an

increased frequency of the mitochondrial T16189C polymorphism, a putative genetic risk factor for dilated cardiomyopathy. We compared the histological, virological and genetic findings of patients with HIV-affected cases with different types of controls, as outlined in chapters 3-6.

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CHAPTER 2

MATERIALS AND METHODS

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2.1 STUDY DESIGN

A case-control study investigating the histological, virological and genetic determinants of HIV-associated cardiomyopathy was established in the Cardiac Clinic, Groote Schuur Hospital (GSH), Cape Town in April 2002 (The HIVAC study). Histological and virological characteristics of HIV-associated cardiomyopathy were examined by endomyocardial biopsy, whereas the genetics sub-study was based on genomic deoxyribonucleic acid (DNA). The endomyocardial biopsy study was designed to obtain myocardial tissue for histological and virological analyses to determine the role of (1) myocarditis, and (2) cardiotropic viruses in the pathogenesis of HIV-associated cardiomyopathy. The genetic sub-study sought to establish whether patients with HIV-associated cardiomyopathy had a greater frequency of the mitochondrial T16189C polymorphisms compared to HIV positive people without cardiomyopathy. The study had been approved by the University of Cape Town Research Ethics Committee (REC REF 016/2002).

The participants in this study were recruited over a three year period from Groote Schuur Hospital and affiliated institutions in the Western Cape. The cases, who were recruited consecutively, were HIV positive patients with new-onset echocardiographically-confirmed diagnosis of dilated cardiomyopathy or left ventricular dysfunction that was otherwise unexplained. Patients with secondary causes of cardiomyopathy or left ventricular dysfunction (such as hypertension, valvular heart disease, diabetes, coronary artery disease, or sepsis) were excluded from the study.

We used different types of controls for the endomyocardial biopsy sub-study (i.e., for histological and virological studies) as compared to the genetics sub-study. Control endomyocardial biopsy samples were obtained from HIV negative patients who were undergoing routine

endomyocardial biopsy in the Cardiac Catheterisation Laboratory at Groote Schuur Hospital, either for the investigation of idiopathic dilated cardiomyopathy or for monitoring of rejection in heart transplant recipients. The heart transplant recipients served as positive controls for immunosuppression and myocarditis, whereas the idiopathic dilated cardiomyopathy cohort acted as negative controls for immunosuppression and myocarditis.

Three control groups were used for the genetics sub-study. The first group included HIV positive people with echocardiographically normal hearts; the controls were recruited from ambulant patients who were attending the Infectious Disease Clinic at Groote Schuur Hospital and were matched with the cases for black African ancestry. The second control group included HIV negative people with echocardiographically diagnosed dilated cardiomyopathy; these controls were recruited from the Cardiac Catheterization Laboratory at Groote Schuur Hospital. The third control group included individuals with no HIV or dilated cardiomyopathy. They represented the South African background population and were obtained from control DNA samples in the Genetics laboratory.

2.2 HISTOLOGY SUB-STUDY

2.2.1 Subjects

We compared the histological changes of patients with HIV-associated cardiomyopathy (n=15) with those of HIV negative patients with idiopathic dilated cardiomyopathy (n=10), and heart transplant recipients (n=15). Due to tissue inadequacy not all the biopsy samples could be used. The discrepancies will be explained in the relevant chapters (chapters 3-6).

2.2.2 Endomyocardial biopsy

The endomyocardial procedure was performed via the right internal jugular vein under fluoroscopic control with an Olympus bioprobe (Tecmed) according to the Stanford technique (Caves 1973). Biopsies were obtained from the middle-distal portion of the right ventricular septum for histological, immunohistochemical, and virological analysis and generally consisted of three to four samples of 1.5-3 mm³. After extraction of the tissue fragment the cardiologist removed the specimen gently with a needle off the biopsy catheter and placed it in three different fixatives: formalin, glutaraldehyde and liquid nitrogen and stored. The samples were subjected to light microscopy, immunohistochemistry, electron microscopy, and fluorescent microscopy by the author under the supervision of two experienced anatomical pathologists (Drs H Wainwright and C Maske) in the Division of Anatomical Pathology at the University of Cape Town.

2.2.3 Light microscopy

2.2.3.1 Protocol

The myocardial biopsy samples for histology were fixed in 10% buffered formalin and embedded in paraffin. The formalin was at room temperature to prevent contraction band artifact. Four serial sections of 4 to 5 µm thickness were cut on a microtome and then mounted on glass slides. The endomyocardial biopsy sections on slides were stained with hematoxylin and eosin (H & E) for histomorphological characterization (i.e., cellular infiltrates, myocyte necrosis, myocyte hypertrophy, nuclear abnormalities, and interstitial fibrosis) and viewed under high power field (magnification, X40) with use of a microscope with wide-field, 10X eyepieces (diameter, 550µm; area, 2.4µm by 105µm; Olympus BH-2, Hamburg, Germany). Specific stains (i.e., Brown and Brenn, Giemsa,

Ziehl-Neelsen, and periodic acid-Schiff) were used to identify bacteria, protozoa, mycobacteria, and fungi. The tissues were examined together with two pathologists who were blinded to the clinical data. We looked for evidence of acute myocarditis (according to the Dallas criteria [see below]), cellular vacuoles, vasculitis, apoptotic myocytes, and variation in nuclear staining, and assessed nuclear size and shape.

2.2.4 Immunohistochemistry

Immunohistochemistry was used to assess the inflammatory infiltrate of myocarditis or acute rejection. Immunoperoxidase staining of serial sections of the endomyocardial biopsy was used for histologic analysis and was performed with monoclonal antibodies to cellular elements CD3, CD4, CD8, and CD68. Formalin fixed tissues were immunostained by using an Envision Double Stain System (DAKO, Carpinteria, California) after antigen retrieval, as described by Berschoner (1990). The antibodies were obtained from DAKO (Carpinteria, California) and Novocastra (Southern Cross Biotechnologies, South Africa). Tonsil tissue was used as a positive control for CD3, CD4, CD8 and CD68 immune cells.

2.2.4.1 Protocol

The slides were placed on a heating block for 5 minutes and then placed in a series of Coplin jars filled with xylol (x1) 100%, alcohol (x2), and 96% alcohol (x2). The slides were then pressure cooked in either EDTA or citrate buffer for two minutes to open the antigenic sites. Endogenous peroxidases were then inactivated by placing the sections in methanol and 3% hydrogen peroxide for 15 minutes and were then placed in water. Sections were then stained with goat serum for 5 minutes in a humidity chamber. Then the primary antibody was applied to all sections for approximately one hour and left in the humidity

chamber. The secondary antibody was then applied and left to stain for 30 minutes. The sections were then stained with the Dab substrate, which is a chromogen. The sections were then placed in copper sulphate for 5 minutes, Scotts water for 15 seconds and hematoxylin for 30 seconds. The slides were then rehydrated in a series of 96% alcohol and xylol Coplynn jars. Sections were then mounted with Entelan onto the slides and viewed.

2.2.5 Diagnosis of myocarditis

There are several histological criteria for the diagnosis of myocarditis including the Dallas and Marburg criteria. The Dallas criteria are centered on the finding of inflammatory cells and/ myocytolysis in order to diagnose acute or borderline myocarditis. The Marburg criteria on the other hand, define an abnormal lymphocytic infiltrate as a finding of more than 14 lymphocytes per high-power field (magnification, X40) with use of a microscope with wide-field, 10X eyepieces (diameter, 550 μ m; area, 2.4 μ m by 105 μ m; Olympus BH-2, Hamburg, Germany). The amount and distribution of fibrosis is described as none (grade 0), mild (grade 1), moderate (grade 2), or severe (grade 3). We used a quantitative method for counting the number of cells per cubic millimeter (similar to the Marburg criteria) in combination with the Dallas criteria. We compared our results to both of these criteria in order to determine the best method for diagnosing myocarditis. Localization of fibrosis was described as endocardial or interstitial.

2.2.6 Transmission Electron Microscopy

Nuclei of cardiac myocytes were studied by transmission electron microscopy for evidence of ultrastructural abnormalities, chromatin herniation, chromatin clumping, loss of peripheral heterochromatin,

irregularities in nuclear lamina and disorganisation of nuclear pore complexes and compared with control samples.

2.2.6.1 Protocol

The tissues were washed with PBS and fixed with 3% glutaraldehyde, buffered with 0.1M sodium phosphate or sodium cacodylate salts (pH 7.4) for 1 h at 25°C, stored in a 0.1M sodium cacodylate buffer containing 0.2M sucrose, postfixed in 1% osmium tetroxide in a 0.1M sodium cacodylate buffer for 1 h at 4°C, dehydrated in a graded series of ethanol and embedded in Epon. Ribbon sections were then cut with a glass cutter and the sections placed on copper grids for viewing under an electron microscope.

2.2.7 Fluorescent Microscopy

We used an antibody that specifically recognises the Rce1-processed form of lamin B1, thereby circumventing the need for expensive biochemical enzyme assays of Rce1 activity (Maske 2003); this antibody (mouse monoclonal antibody 8D1) was available as part of the collaboration with Dr CP Maske. Commercial antibodies to be used in this study included mouse anti-lamin () A/C (Novocastra Ltd) and goat polyclonal anti-lamin B1 (Santa Cruz Biotech), both of which had been extensively characterised during his previous work.

Serial sections were cut from myocardial tissue and labeled with anti-lamin antibodies using standard immunohistochemical techniques. Rce1-mediated endoproteolysis of lamin B1 was measured by labeling with antibody 8D1, a mouse monoclonal antibody that has been previously described. An internal control exists in all sections in the form of non-cardiac myocyte cells including nuclei of endothelial cells, peripheral blood cells, fibroblasts and endocardium. Intensity of labeling

of these non-myocyte cells was used in normalisation of signals. Ubiquitous expression of B-type lamins and expression of A-type lamins in terminally differentiated cells allows such normalisation to be performed.

2.2.7.1 Protocol

The slides were labeled and placed on a heating block for 5 minutes to heat-fix the tissues onto the slides. The slides were then placed in a series of Coplin jars filled with xylol (x1) 100%, alcohol (x2), and 96% alcohol (x2). The slides were placed in the appropriate buffer for antigen retrieval and pressure cooked for 2 minutes. Slides were then blocked with donkey serum or gelatine for 30 minutes at room temperature. The serum was drained and the primary antibody (C20 goat x laminB1(1:100)) was placed onto the slide and incubated in a humidity chamber for 1 hour at room temperature. The slides were then washed (x3) with 1xPBS and stained with Sudan black to stop autofluorescence. The slides were again washed with 1xPBS (x3). The secondary antibody (Donkey x goat-A**488) was then placed onto the slides and incubated at room temperature for 1 hour. The slides were then again washed in 1xPBS (x3) and then briefly rinsed in water. The slides were then mounted in DAPI/M, stood for 10 minutes and then viewed.

2.3 VIROLOGY SUB-STUDY

2.3.1 Subjects (see 2.2.1 above)

The virological studies were performed on the same subjects who underwent endomyocardial biopsy for the histology study, as indicated in section 2.2.1.

2.3.2 Protocol

For each patient in the three cohorts, one biopsy was taken at the time of cardiac catheterization and immediately snap frozen (Appendix 1) for the necessary analysis. To further corroborate the histology diagnosis in Chapter 3, immunoperoxidase staining on serial sections of the same paraffin-embedded tissue used for histology was performed with monoclonal antibodies to cytomegalo- , Epstein-Barr-, Herpes Simplex-, parvo-, adeno, entero- and HIV-1 viruses (Novocastra, Southern Cross). We compared the frequency of positive staining in the myocardium for the various viruses in patients with HIV-associated cardiomyopathy, idiopathic dilated cardiomyopathy, and heart transplant recipients, using an Olympus BH-2 microscope with wide field 10X eyepiece (high power field X400).

2.3.3 DNA and RNA extraction from endomyocardial biopsy and blood samples

The genetic and virology investigations were carried out in the Division Human Genetics at the University of Cape Town using two methods. In the first method a commercial kit (Qiagen, DNA blood minikit, Southern Cross, USA) was used to isolate total DNA and RNA from blood samples, while only RNA was extracted from the biopsy tissues using the same kit. In the second method, endomyocardial tissue samples were first homogenized in RNazol by use of disposable RNase-free pestles. Total RNA and genomic/viral DNA were isolated simultaneously from patient specimens and resuspended in 25 mL of diethyl pyrocarbonate (DEPC)-treated water using the DNA blood minikit. HIV-1 DNA, adenovirus DNA and enterovirus RNA, isolated from infected cultured cells, were used as positive viral controls for PCR analysis after nucleic acid extraction.

2.3.4 cDNA synthesis by reverse transcription

To amplify RNA species, cDNA is synthesized from RNA templates by reverse transcription and then the cDNA is enzymatically amplified. Since enteroviruses are RNA viruses, reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate these viruses.

Serial dilutions of enterovirus RNA was made (1:10 / 1:100 / 1:1000) as it was realized that the isolated RNA was of too high a concentration to amplify. Isolated RNA was treated with DNAase (Roche Diagnostics) to digest all genomic DNA that might be present in the sample. Reverse transcriptase-PCR (iScript Kit, Bio-RAD) was used to amplify 155 nucleotide portion of the 5' noncoding region of the genome of enteroviruses (Zoll 1992). To increase the sensitivity a semi-nested reaction was used (Yeats 2003). Appropriate negative and positive controls were included.

2.3.4.1 Protocol

To synthesize the cDNA the following reagents were used in one reaction of 20ul : 1ul iScript, 4ul 5 X iScript Buffer, 5ul Nuclease free water and 10ul RNA. The following cycling parameters were used: 64°C for 5 minutes, 42°C for 30 seconds, 25°C for 10 minutes, 42°C for 45 minutes and 85°C for 5 minutes.

2.3.5 Polymerase Chain Reaction (PCR)

Primer pairs (Table 1) for both DNA and RNA viruses were designed to detect classes of viruses known to be implicated as causes of viral myocarditis (i.e., cardiotropic viruses). The enteroviral primer sequences were selected from highly conserved parts of the 5'-noncoding region (Zoll 1992); adenovirus-specific primers were designed to amplify the genomic sequence of adenoviruses encoding the hexon protein, amplifying all

adenovirus serotypes for which sequence data were available in GenBank (Pauschinger 1999). The primer sequences for the other viruses were obtained from the various papers (Table 1).

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Table 1 PCR primer sets used for amplification

	SEQ.F	SEQ.R	Amplicon	SIZE (bp)	References
CMV	5'-GAG GCT ATT GTA GCC TAC ACT TTG G -3'	5'-CTC TAT CTC AGA CAC TGG CTC AGA C -3'	1		
	5'-CCA CCC GTG GTG CCA GCT CC -3'	5'-CCC GCT CCT CCT GAG CAC CC -3'	2	160bp	Ishigaki 1991
EBV	5'-AAG GAG GGT GGT TTG GAA AG -3'	5'-AGA CAA TGG ACT CCC TTA GC -3'	1		
	5'-ATC GTG GTC AAG GAG GTT CC -3'	5'-ACT CAA TGG TGT AAG ACG AC -3'	2	209bp	Clinque 1993
HSV	5'-GT(GC) AA(AG) CAG TAC GGC CCC GAG -3'	5'-GGA TGT CGC G(GA)T AGC TCA G -3'	1		
	5'-ATC AAC TTC GAC TGG CCC TTC-3'	5'-CCG TAC ATG TCG ATG TTC ACC-3'	2	179bp	Lakeman 1995
PARVO	5'-TAT GCT TAC TTA ACA GTA GG -3'	5'-AAT TGG CCC ACT TTG TGG -3'	1		
	5'- GCC TTA GCA CAG GTA CCT CT -3'	5'-CAT CCT CCT AAG GCT GCA AAC-3'	2	397bp	Heegaard 2001
ADENO	5'-ACTACAAYATTGGCTACCAGG -3'	5'- CAAAACATAAAGAAGKGTGGGC -3'	1	440bp	
	5'-AACTTCCAGCCCATGAGCMG -3'	5'- CTCAAAAGTCATGTCBAGCGC -3'	2	330bp	Pauschinger 1999
ENTERO	5'-CAA GCA CTT CTG TTT CCC CGG -3'	5'- ATT GTC ACC ATA AGC AGC CA -3'	1		
	5'- ATT GTC ACC ATA AGC AGC CA -3'	5'- TCC TCC GGC CCC TGA ATG CG -3'	2	155bp	Zoll 1992
HIV-1	5' ATA ATC CAC CTA TCC CAg TAg gAg AAA T -3'	5'- TTT ggT CCT TgT CTT ATg TCC AgA ATg C-3'	1		Rayfield 1988

2.3.4.1 Protocol

Following DNA extraction (DNA viruses) and cDNA synthesis (RNA viruses) the following PCR protocol was used: **primary PCR per 25ul reaction:** 200uM dNTP's; 1.5mM MgCl₂; 10x Invitrogen buffer; 0.5U Taq polymerase; 20nM forward primer, 20nM reverse primer; 100ng/ul DNA; make up to 25ul with sterile distilled water. **Secondary PCR per 25 ul reaction:** 200uM dNTP's; 1.5mM MgCl₂; 10xKCl buffer; 0.5U Taq polymerase; 20nM forward primer, 20nM reverse primer and 2ul amplicon produced in the primary reaction; make up to 25ul with sterile distilled water.

The cycling parameters were as follows: 95°C for 4 minutes; 95°C for 1 minute, annealing temperature for 45 seconds, 68°C for 1 minute (30cycles); 72°C for 7 minutes. These parameters were followed for both primary and secondary PCR protocols. All samples were amplified with a simultaneous positive and negative control (i.e., reaction mixture minus template nucleic acid) for the virus analyzed. If a band was visualized in the negative control lane, the PCR sample was considered contaminated and the sample reanalyzed or re-extracted and analyzed. All samples were analyzed without prior knowledge of clinical or serological data for each patient, and all PCR-positive samples were required to have duplicate results. Control PCR amplification to verify the presence of amplifiable nucleic acid extracted from each sample was performed using primers designed to amplify cellular nucleic acid; β -globin primers were chosen from the paper published by Bell *et al* 1993. If β -globin primers failed to amplify the appropriate 135bp amplicon, the sample was re-extracted or excluded.

The PCR products were separated on a 1.5% agarose gel and the ethidium bromide stained bands were visualised under UV light.

Viruses (cytomegalo- , Epstein-Barr-, herpes simplex-, parvo-, adeno, entero- and HIV-1 viruses) used as positive controls for PCR or

immunohistochemistry were obtained from cell cultures grown in the Division of Medical Virology at Groote Schuur Hospital. Enterovirus and adenovirus positive cultures were obtained from the heart of a histologically confirmed case of myocarditis as well as cultures obtained from our Medical Virology laboratory. Culture positive cells were embedded in paraffin blocks, serial ribbon sections cut and placed on glass slides. Viral genomes were extracted according to RNA/DNA extraction kit (Qiagen, Southern Cross).

2.3.6 Immunohistochemistry (see 2.2.4 above)

Immunohistochemistry was performed according to the same methods outlined in 2.2.4 above. Briefly, formalin-fixed tissues were immunostained by using an Envision Double Stain System (DAKO, Carpinteria) after antigen retrieval. The tissue was scrutinized by two independent pathologists who were blinded to the clinical details.

2.4 GENETICS SUB-STUDY

2.4.1 Study design

This was a case-control study of incident cases of HIV-associated cardiomyopathy (HIV+DCM+) and HIV positive non-cardiomyopathy controls (HIV+DCM-) as well as a replication study of HIV negative cases with idiopathic dilated cardiomyopathy and the background population. These groups were matched by ethnicity. Spurious associations may arise in genetic association studies due to the inclusion of a population sub-group having different allele frequencies for the variant under study (Keavney 2000). To control for this confounder, the cases and controls were drawn from people of black African ancestry.

The original sample size calculation for this study was based on comparing the two proportions that present the exposure to the

mitochondrial DNA T16189C polymorphism (also called the 'OriB' variant) among the controls and the cases. The estimates of these proportions were based on the results obtained for the Black African population in the Khogali study where 45.5% of cases and 15.7% of controls presented with the OriB variant (Khogali 2001). Because of the suspected higher incidence of the variant among the cases than among the controls, no benefit was considered to be possible from an unequal division of cases and controls. Hence we adopted a 1:1 ratio of cases and controls. The significance level was fixed at 95%. To obtain 95% confidence, with 80% power for a two-sided hypothesis and based on exposure rates of 46% among cases and 16% among controls, we needed to recruit 43 cases and 43 controls.

The DNA was analysed for the OriB variant using standard PCR (see 2.4.2 below) and sequencing (see 2.4.3 below) of the D-loop region (nucleotides 15894–16401) in both the forward and reverse directions. The primer pair published in Khogali (Table 2) was used for mitochondrial PCR amplification. The resultant amplicon was sequenced on the ABI3100 and analysed.

DNA was extracted according to the protocol in Chapter2, section 2.3.3.

2.4.2 Polymerase Chain reaction (PCR)

2.4.2.1 Protocol

The primer pairs published in the Khogali paper (Table2) were used for OriB variant amplification (Khogali 2001).

Table 2 Primers (with M13 tails) used for polymerase chain reaction (PCR) of the T16189 mitochondrial segment

	Light chain (forward prime)	Heavy chain (reverse primer)	Fragment size
D-loop segment 1	5'TGTAAAACGACGGCCAGTC TCAAATGGGCCTGTCCTTG 3'	5'CAGGAAACAGCTATGACC TTGATTCACGGAGGATGGT G 3'	502bp

The following PCR protocol was used: **primary PCR per 25ul reaction:** 200uM dNTP's; 1.5mM MgCl₂; 10xKCl buffer; 0.5U Taq polymerase; 20nM forward primer, 20nM reverse primer; 100ng/ul DNA; make up to 25ul with sterile distilled water. **Secondary PCR per 25 ul reaction:** 200uM dNTP's; 1.5mM MgCl₂; 10xKCl buffer; 0.5U Taq polymerase; 20nM forward primer, 20nM reverse primer and 2ul amplicon produced in the primary reaction; make up to 25ul with sterile distilled water. The cycling parameters were as follows: 95°C for 4 minutes; 95°C for 1 minute, annealing temperature for 45 seconds, 68°C for 1 minute (30cycles); 72°C for 7 minutes. These parameters were followed for both primary and secondary PCR protocols.

All samples were amplified with a simultaneous positive and negative control (i.e., reaction mixture minus template nucleic acid). If a band was visualized in the negative control lane, the PCR sample was considered contaminated and the sample reanalyzed or re-extracted and analyzed. All PCR-positive samples were required to have duplicate results.

The PCR products were separated on a 1.5% agarose gel and the ethidium bromide stained bands were visualised under UV light.

2.4.2 Sequencing

2.4.3.1 Protocol

The correct band was cut out from the gels and cleaned using the Qiagen gel cleanup kit (Southern Cross, USA). The following sequencing protocol was used per reaction: 3ul termination mix, 4ul sequencing buffer, 4ul of a 0.8mM forward or reverse primer, 2ul cleaned up product, 7ul distilled water.

The following cycling parameters were used for sequencing: 64°C for 10 minutes, 51°C for 45 minutes for 25 cycles. This product was purified through a centricep column (Separations Scientific, White head

Scientific). The columns were prepared as per the instructions in the manual.

The cleaned up sequencing products were run on an ABI3100.

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CHAPTER 3

HISTOLOGICAL FEATURES OF HIV-ASSOCIATED CARDIOMYOPATHY

CHAPTER 3: HISTOLOGICAL FEATURES OF HIV ASSOCIATED CARDIOMYOPATHY

3.1 INTRODUCTION

The literature was reviewed on the role of myocarditis in the context of HIV-associated cardiomyopathy and is presented through a series of studies conducted before and after the formulation of the Dallas criteria for myocarditis in order to ascertain an understanding of the disease and its evolution over the past two decades.

The association of HIV infection with cardiac pathology was recognised in the early stages of the HIV epidemic. Most of the studies reporting on the association of HIV with cardiac abnormalities have been carried out in North America (17/23), with only three in Italy (3/23), one in France (1/23), one in Scotland (1/23) and one in the Democratic Republic of Congo (1/23).

In the mid 1980's, three autopsy studies brought HIV-associated cardiomyopathy sharply into focus. These studies reported on the clinico-pathological features of HIV-associated cardiomyopathy in the period before the promulgation of the Dallas criteria (Fink 1984, Cammarosano 1985, Cohen 1986). These studies used light microscopy, viral cultures, Western blot and serology to identify patients with myocarditis. Cammarosano and Lewis (1985) conducted the first autopsy study which positively identified myocarditis in HIV-associated cardiomyopathy cases using only light microscopy; *Cryptococcus neoformans* was the only pathogen identified (Cammarosano and Lewis 1985). It was not until Cohen (1986) observed three fatal cases of AIDS in which there were striking clinical, echocardiographic, and morphologic findings of dilated cardiomyopathy, that the entity of HIV-associated cardiomyopathy (HIVAC) was first recognised. These three autopsy cases documented the occurrence of clinically important myocardial compromise due to dilated cardiomyopathy in association with AIDS. In each case, recurrent opportunistic infection (in the mouth and lungs)

had been present for over six months before rapidly fatal acute cardiac illness became clinically evident four to eight weeks prior to death. At autopsy, two patients revealed generalized cardiomyopathy with focal lymphocytic myocarditis which, in the absence of evidence of bacterial or parasitic infection, was suggestive of a viral aetiology; HIV-1 was positively identified from heart tissue by Western Blot and viral culture (Cohen 1986). This suggested a potential role for HIV as an aetiological agent in cardiomyopathy.

However, controversy surrounded the diagnosis of myocarditis, with its wide variety of aetiologies, its heterogeneous clinical presentations as well as the lack of uniformity in diagnostic classification. The methods used in previous studies (light microscopy, viral culture, Western blot, and serology) appeared to definitively identify only a portion of patients with myocarditis; additional methods were needed to improve the diagnostic accuracy in cases of suspected myocarditis. To resolve this problem, the Dallas criteria were introduced in 1986.

The Dallas criteria, served as the first attempt to develop standardized diagnostic guidelines for the histopathological classification of myocarditis. A group of eight pathologists with experience and interest in the interpretation of endomyocardial biopsies met in March 1984, in conjunction with the American College of Cardiology meeting in Dallas, Texas. The goals of the group were to (1) define myocarditis from the morphologic standpoint; (2) develop histopathologic criteria for the diagnosis of myocarditis; (3) establish a simple, reproducible working classification; (4) outline the problems and pitfalls related to establishing the diagnosis of myocarditis; (5) assess the applicability and reproducibility of the classification system; and (6) make the information available to pathologists and clinicians (Aretz 1986). The results of the proceedings are summarised in Table 3.

Table 3 Classification of myocarditis: Dallas vs Marburg criteria

<u>Dallas criteria (Aretz et al 1987)</u>	<u>Marburg criteria (URL1)</u>
First biopsy	First biopsy
(1)Active myocarditis	(1)Acute (active) myocarditis
- lymphocytic infiltrate with myocytolysis; with or without fibrosis	-lymphocytic infiltrate of ≥ 14 leukocytes/mm ² with myocyte necrosis/degeneration; with or without fibrosis
(2)Borderline myocarditis	(2)Chronic myocarditis
-lymphocytic infiltrate with no myocytolysis	-lymphocytic infiltrate of ≥ 14 leukocytes/mm ² myocyte necrosis/degeneration not evident; with or without fibrosis
(2)No myocarditis	(3)No myocarditis
-no lymphocytic infiltrate, no myocytolysis	-lymphocytic infiltrate of < 14 leukocytes/mm ²
Subsequent biopsies	Subsequent biopsies
Ongoing (persistent) myocarditis with or without fibrosis	Ongoing (persistent) myocarditis: features of an acute or chronic myocarditis (i.e., criteria 1or 2)
lymphocytic infiltrate with myocytolysis	
Resolving (healing) myocarditis with or without fibrosis	Resolving (healing) myocarditis: criteria 1 or 2 but immunological process is sparser than in the first biopsy
lymphocytic infiltrate without myocytolysis	
Resolved (healed) myocarditis with or without fibrosis	Resolved (healed) myocarditis: corresponds to the Dallas classification; lymphocytic infiltrate of < 14 leukocytes/mm ²
No lymphocytic infiltrate no myocytolysis	

Over the past two decades, twenty three studies in total have studied the characteristics of HIV-associated cardiomyopathy.

Table 4 is a summary of these results, describing a variety of cardiac abnormalities and histopathological findings in AIDS patients infected with HIV-1.

Table 4 Summary of histological studies on HIV-associated cardiomyopathy on human tissue

<u>Study</u>	<u>Type</u>	<u>Country</u>	<u>Technique</u>	<u>Pathogens</u>	<u>Sample size</u>	<u>ARV</u>	<u>Cardiac pathology</u>	<u>Dallas or Marburg criteria</u>	<u>Cardiomyopathy/myocarditis</u>
<u>Studies prior to the Dallas Criteria</u>									
Fink <i>et al</i> 1984	Autopsy	Pennsylvania	Echocardiography,	No opportunistic infections ID	N=15	Not stated	2/4 enocard 1/4 pericard	Not stated	15HIV+DCM+
Cammarosano and Lewis 1985	Autopsy	Los Angeles	Light microscopy	<i>C. neoformans</i> (+)1/1	N=10	Not stated	4/10 Kaposi's	Not stated	10DCM+HIV+, 1MC
Cohen <i>et al</i> 1986	Autopsy	Washington	Light microscopy, Electron microscopy, Western Blot, viral culture	WB HIV-1(+) HIV+ id., cult (+) No opportunistic infections	N=3	Not stated	1 PM not done	Not stated	3 DCM+HIV+, 2MC
<u>Studies Post the Dallas Criteria</u>									
Roldan <i>et al</i> 1987	Autopsy	Miami	Light microscopy	<i>T. gondii</i> (6/17) 11/17 unknown	N=54	Not stated	30/54 path. changes	Not stated	54 DCM+HIV+, 17MC
Calabrese <i>et al</i> 1987	EMB	Ohio	Light microscopy, Electron microscopy, viral culture	HIV-1+ culture No opportunistic infections ID	N=1	Not stated	No MC	Not stated	1HIV+DCM+, 0 MC
Anderson <i>et al</i> 1988	Autopsy	Maryland	Light microscopy	9/37 (mixed) CMV(+)	N=71	Not stated	34/71 MC-	Dallas ≥5 lymphs	71HIV+, 19HIV+DCM+, 37MC
Reilly <i>et al</i> 1988	Autopsy	Maryland	Light microscopy	CMV (2/26) No opportunistic infections <i>T.gondii</i> (1/9)	N=58	Not stated	32/58 MC- 15/26 cardiac abnormal 33/58 Kaposi's 2/26 Kaposi	Dallas ≥5 lymphs	58 DCM+HIV+, 26 MC
Baroldi <i>et al</i> 1988	Autopsy	Italy	Light microscopy	Sarcosporidium(1/9) <i>C.neoformans</i> (1/9) CMV(+)	N=26	Not stated	2/26 \infarct necrosis 13/26 Coagul. Mycytol	Dallas	26HIV+, 9/26 DCM- HIV+ MC+,9/26 DCM+HIV+ MC+

<u>Study</u>	<u>Type</u>	<u>Country</u>	<u>Characteristics</u>	<u>Pathogens</u>	<u>Sample size</u>	<u>ARV</u>	<u>Cardiac pathology</u>	<u>Dallas or Marburg criteria</u>	<u>Cardiomyopathy/myocarditis</u>
<u>Studies Post the Dallas Criteria (cont.)</u>									
Lafont <i>et al</i> 1988	Autopsy	French	Light microscopy	CMV (2/14) <i>T.gondii</i> (5/14) <i>C.neoformans</i> (3/14) <i>C.albicans</i> (2/14) <i>M.tuberculosis</i> (1/14)	N=137	Not stated	29/56 CM- 13/56 (mild) CM 14/56 (severe)CM	Not stated	27 HIV+DCM+, 56MC+
Levy <i>et al</i> 1988	EMB	Washington, DC	Light microscopy, viral culture	viral cult (-) No opportunistic infections ID ISH HIV-1(-)	N=2	Not stated	All MC	Not stated	2/ HIV+DCM+, 2 MC
Dittrich <i>et al</i> 1988	EMB	California	Light microscopy, Electron microscopy, <i>in situ</i> hybridization, viral culture	HIV+ cult serum CVB3+ No opportunistic infections ID	N=1	Not stated	No MC	Not stated	HIV+DCM+, 0 MC
Corallo <i>et al</i> 1988	Autopsy	Italy	Light microscopy, Echocardiography	Not stated	N=102	Not stated	55/102 tchycar.&□□LV 42/102□contracting LV 39/102 pericardial effusion 4/102 valvular endocard.	Dallas	97HIV+DCM+, 25MC+
Flomenbaum <i>et al</i> 1989	Autopsy	New York	Light microscopy, Electron microscopy, PCR, <i>in situ</i> hybridization, Southern blot	PCR (HIV-1+)(3/4) ISH HIV-1(+) 3/4 Southern blot (HIV-1+) CMV(+)	N=4	Not stated	0/4 DCM 1/4 Infective endocard.	Dallas	4HIV+, 0MC
Himelman <i>et al</i> 1989	Prospective	California	Echocardiography	CMV(+)	N=70	Not stated	7/70 pericard effusn 4/70 pleural effusion 1/70 mediastinal mass	Not stated	70HIV+, 8/70 HIV+DCM+

<u>Study</u>	<u>Type</u>	<u>Country</u>	<u>Characteristics</u>	<u>Pathogens</u>	<u>Sample size</u>	<u>ARV</u>	<u>Cardiac pathology</u>	<u>Dallas or Marburg criteria</u>	<u>Cardiomyopathy/myocarditis</u>
<i>Studies Post the Dallas Criteria (cont.)</i>									
Wilkins <i>et al</i> 1989	EMB	Texas	Light microscopy, Electron microscopy, Western blot	WB (HIV-1(+) No opportunistic infections ID	N=1	1/1	Resolved MC	Not stated	1 HIV+DCM+, 0 MC
Grody <i>et al</i> 1990	Autopsy	California	Light microscopy, <i>in situ</i> hybridization	HIV-1(+) (6/22)	N=23	Not stated	DCM status not mentioned	Not stated	23HIV+
Beschorner <i>et al</i> 1990	EMB	Georgia	Light microscopy, Immunohistochemistry, viral culture	No opportunistic infections ID	N=16	Not stated	5/16 no MC	Dallas ≥10 lymphs	16HIV+DCM+, 11 MC
Parravicini <i>et al</i> 1991	Autopsy	Italy	Light microscopy, Immunohistochemistry	HIV-1(+) (2/10) CMV+ (5/10) Pathogens not ID (0/34)	N=34	Not stated	4/34 mild LV dilatn 8/34 minor ECG abnor	Dallas	34HIV+ (paraffin embed sectns)(20MC), 10 frozen cardiac sectns(6/10MC+), 4HIV+DCM+ 10HIV+DCM-, 5 HIV+DCM+, 2/5HIV+DCM+(PCR MC+), 6/10HIV+DCM-(PCR MC+)
Rodrigues <i>et al</i> 1991	EMB	Washington, DC	Light microscopy, Electron microscopy, Immunohistochemistry, Western blot, PCR, viral culture	HIV-1 (+) (2/5)	N=15	12/15	3/5HIV+DCM+(MC-) 4/10HIV+DCM-(MC-)	Dallas	296HIV+, 13HIV+DCM+
Currie <i>et al</i> 1994	Prospective	Edinburgh	Echocardiography	NO HISTOLOGY	N=296	Not stated	13 DCM 12 RV dysfunction 19 borderl LV dysfunctn	Not stated	296HIV+, 13HIV+DCM+
Herskowitz <i>et al</i> 1994	EMB	Georgia	Light microscopy, <i>in situ</i> hybridization, viral culture	<i>C.neoformans</i> (2/37) CMV+ HIV-1(+)	N=37	21/33	2/37(hypersensitiv. MC) 16/33 (MC-)	Dallas ≥10 lymphs	37HIV+ DCM+, 17/33 MC

<u>Study</u>	<u>Type</u>	<u>Country</u>	<u>Characteristics</u>	<u>Pathogens</u>	<u>Sample size</u>	<u>ARV</u>	<u>Cardiac pathology</u>	<u>Dallas or Marburg criteria</u>	<u>Cardiomyopathy/myocarditis</u>
<i>Studies Post the Dallas Criteria (cont.)</i>									
Longo-Mbenza <i>et al</i> 1998	Autopsy	Congo	Light microscopy, viral culture, Echocardiography, PCR	<i>T.gondii</i> (3/16) <i>C.neoformans</i> (3/16) <i>M.avium</i> (2/16) HIV-1 (+)(8/16)	N=157	Not stated	32/157 pericardial dise 22/157 concentr hypertr 23/157 LV systolic dysf 65/157 LV diast dysf	Not stated	157HIV+, 55DCM+HIV+, 16MC
Pulerwitz <i>et al</i> 2004	EMB	New York	Light microscopy	Not stated	N=171	Not stated	115-1° MC 27 peripartumn 22 HIV MC 7 inflammatory disorders	Dallas	171 MC(1° and 2°) 22/56 HIV+DCM+

LM-light microscopy, EM-electron microscopy, ISH-in situ hybridization , IHC-immunohistochemistry, PCR-polymerase chain reaction, MC-myocarditis, PM post mortem, CMV-cytomegalo virus *Toxoplasma.gondii*, *Cryptococcus neoformans*, *Candida albicans*, *Mycobacterium tuberculosis*

Twelve autopsy and eight endomyocardial biopsy studies investigated the characteristics of HIV-associated cardiomyopathy after the formulation of the Dallas criteria. Three of the studies were echocardiographic studies that later went on to study postmortem tissues (Fink 1984, Corallo 1988, Currie 1994).

Eight of the twelve autopsy studies positively identified myocarditis. Anderson and his colleagues conducted the first autopsy study to report myocarditis in HIV-associated cardiomyopathy patients using the Dallas criteria (Anderson 1988). Seven other autopsy studies later confirmed this finding (Roldan 1987, Reilly 1988, Baroldi 1988, Lafont 1988, Corallo 1988, Parravicini 1991, Longo-Mbenza 1998), identifying active or borderline myocarditis which was supported by PCR, *in situ* hybridizations or viral cultures. The Dallas criteria were used in only five of the eight autopsy studies that found myocarditis (Anderson 1988, Reilly 1988, Baroldi 1988, Corallo 1988, Parravicini 1991). One study, using light microscopy (the Dallas criteria), did not observe any myocarditis but they did however detect HIV-1 using PCR and *in situ* hybridization (Flomenbaum 1989).

One of the research highlights in the investigation of HIV-associated cardiomyopathy was carried out in an endomyocardial biopsy series in Washington DC by Levy (1988). This was the first study to document lymphocytic myocarditis in two HIV infected patients with significant cardiac dysfunction; however, viral cultures remained negative (Levy 1988). Four endomyocardial studies carried out later confirmed the finding of Levy *et al.*, demonstrating the high prevalence of myocarditis in endomyocardial biopsy from HIV positive patients with left ventricular dysfunction (Wilkins 1989, Berschorner 1990, Herskowitz 1994, Pulerwitz 2004). Of the five studies that found myocarditis only three had used the Dallas criteria; the other studies did not specify how myocarditis was diagnosed. The three studies that did not find myocarditis (Calabrese 1987, Dittrich 1988, Rodrigues 1991) were (1)

small (i.e., two of the three studies reported on only one case), (2) found HIV-1 in heart tissue using either viral cultures or *in situ* hybridization.

The techniques used to diagnose myocarditis exacerbated the debate, with many researchers reporting contrary findings. In 1990, an autopsy study by Grody provided the first microscopic evidence for the direct infection of the heart by HIV-1 (Grody 1990). As there are no known receptors for HIV on cardiac myocytes, how is HIV -1 able to enter the myocyte? This study was followed by the study by Beschorner who was the first to use immunohistochemistry (a direct detection assay) to identify the nature of the inflammatory cells targeting the myocardium in myocarditis positive HIV-associated cardiomyopathy patients. Then in Rodrigues (1991), using the PCR and a radio-labeled probe in endomyocardial tissue, went on to show the presence of the HIV sequences in myocytes with and without cardiac dysfunction as well as the presence of the HIV antigen in dendritic cells in all patients with cardiac symptoms (Rodrigues 1991).

However, many researchers believed that the definition of inflammation was left open with reference to the Dallas classification, which distinguished by conventional hematoxylin and eosin staining active myocarditis in the first biopsy, versus ongoing (persistent) myocarditis, healing or healed (resolved) in a subsequent biopsy. They felt that, within the context of inflammatory cardiomyopathy, terms such as active/acute or chronic myocarditis, the association between pericardial disease and myocarditis (perimyocarditis), autoreactive or virally induced myocarditis needed further explanation and diagnostic consensus.

Then, in 1995, the World Health Organisation/International Society and Federation of Cardiology Task Force (ISFC, presently World Heart Federation, WHF) on the definition and Classification of Cardiomyopathies formed two expert committees, one with international experts on histopathology and immunohistochemistry and another one

with international experts on the molecular diagnosis of infective or viral cardiomyopathies, which convened in separate sessions in Marburg, Germany (Maisch 2000, URL2).

The two committees proposed several changes in the definition and classification of heart muscle diseases (Maisch 1997). This new definition of dilated cardiomyopathies also comprised inflammatory dilated cardiomyopathy, chronic myocarditis and viral cardiomyopathies. Inflammatory cardiomyopathy was defined as “myocarditis in association with cardiac dysfunction”. Idiopathic, autoimmune, and infectious forms of inflammatory cardiomyopathy were recognized; the committee defined myocarditis as a process characterized by an inflammatory infiltrate of the myocardium. The terminology and criteria that were adopted later became known as the Marburg criteria. To date, none of the international studies published on HIV-associated cardiomyopathy have used the Marburg criteria for diagnosis of myocarditis.

In Africa, there is a paucity of histological studies that have examined the cause of cardiomyopathy in people infected with HIV (Magula and Mayosi 2003). We have identified one report of cardiac histology in 16 HIV-positive patients with cardiomyopathy who were examined post mortem (Longo-Mbenza 1998). All 16 patients with HIV-associated cardiomyopathy had histopathological evidence of acute myocarditis, which was attributed to *Toxoplasma gondii* (3/16), *Cryptococcus neoformans* (3/16), *Mycobacterium avium intracellulare* (2/16) and to HIV in the rest of the series (8/16). The results of this small study raise the prospect that HIV-associated cardiomyopathy may be due to potentially treatable opportunistic infections in up to 50% of cases. By contrast, in Western series, opportunistic viral infections have been implicated in a significant proportion of cases.

We have conducted the first endomyocardial biopsy study to examine the role of myocarditis in the pathogenesis of HIV-associated cardiomyopathy in patients living in Africa.

3.2 METHODS

The methods of selection of the participants, endomyocardial biopsy and histology are described in Chapter 2 (sections 2.1 and 2.2)

The results are presented as semi-quantitative and morphological data. The adequacy of the number of cells quantitated in each individual section was assessed using an average mean analysis. The mean expression levels from different groups was compared using ANOVA and where necessary the log-rank test; where some observations were 0 and it was not possible to log-transform the data, square root transformation of the data was used.

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3.3 RESULTS

3.3.1 Clinical characteristics of study subjects

We compared the histological and immunohistochemical features of 15 cases with HIV-associated cardiomyopathy, 15 heart transplant recipients and 10 cases with idiopathic dilated cardiomyopathy using endomyocardial biopsy samples. A total of nine endomyocardial biopsy samples contained mainly fatty tissue and were considered to be unsuitable for further analysis, and the breakdown of these samples was as follows: HIV-associated cardiomyopathy n=3, heart transplant recipients n=4 and idiopathic dilated cardiomyopathy n=2. Therefore, thirty one patients, consisting of 12 patients with HIV-associated cardiomyopathy, 11 heart transplant recipients and eight with idiopathic dilated cardiomyopathy cases were entered in the final analysis.

The clinical characteristics and echocardiographic measurements are summarized in Table 5.

Table 5 Clinical characteristics of patients with endomyocardial biopsy samples that were analysed in this study

	HIV-associated cardiomyopathy	Heart transplant recipients	Idiopathic dilated cardiomyopathy	P-value
Number of patients	12	11	8	
Mean age-yr ($\pm$ SD)	40.5 (10.7)	47.1 (14.1)	43 (7.35)	0.22
Male-%	58	82	75	0.44
Mean CD4 count - cells/mm ² ($\pm$ SD)	246 (193)	Not applicable	Not applicable	
Mean Viral load(Median)	1 234 835(155 000)	Not applicable	Not applicable	
Mean LVEF- % ($\pm$ SD)	27.9 (6.92)	52.8 (7.7)	26.2 (10.39)	0.0001
Duration of illness in weeks ($\pm$ SD)	19.73 (11.9)	21.64 (13.1)	15.04 (10.4)	0.3

There was no significant difference between the different groups in age, sex, and duration of illness. The poor systolic function of the heart is clearly demonstrated by the diminished left ventricular ejection fraction for both the HIV-associated cardiomyopathy and idiopathic dilated cardiomyopathy cohorts. Consequently, patients with HIV-associated cardiomyopathy and idiopathic dilated cardiomyopathy had a greater frequency of dyspnoea, as indicated by impaired New York Association Functional Class (Figure 3), as compared to the heart transplant recipients who were asymptomatic.

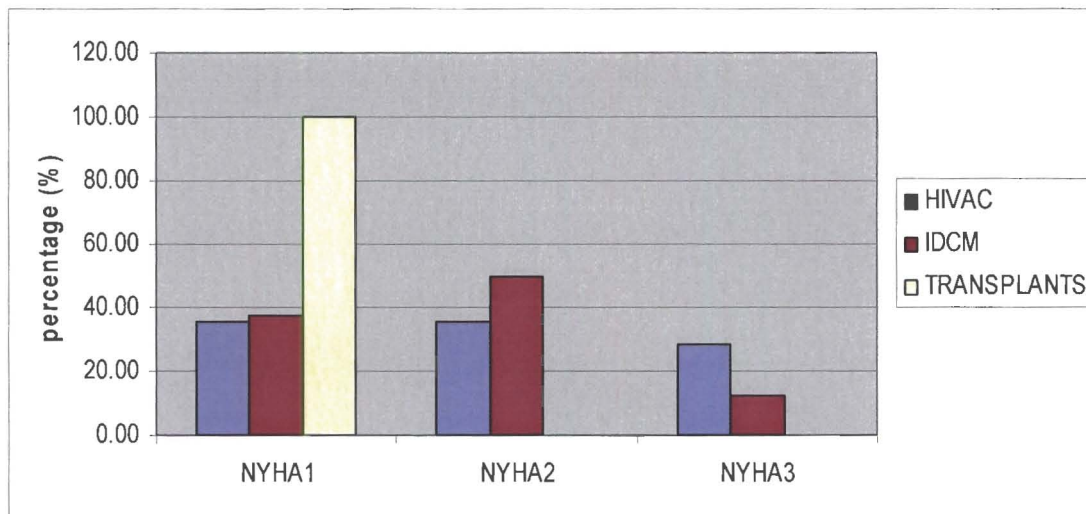


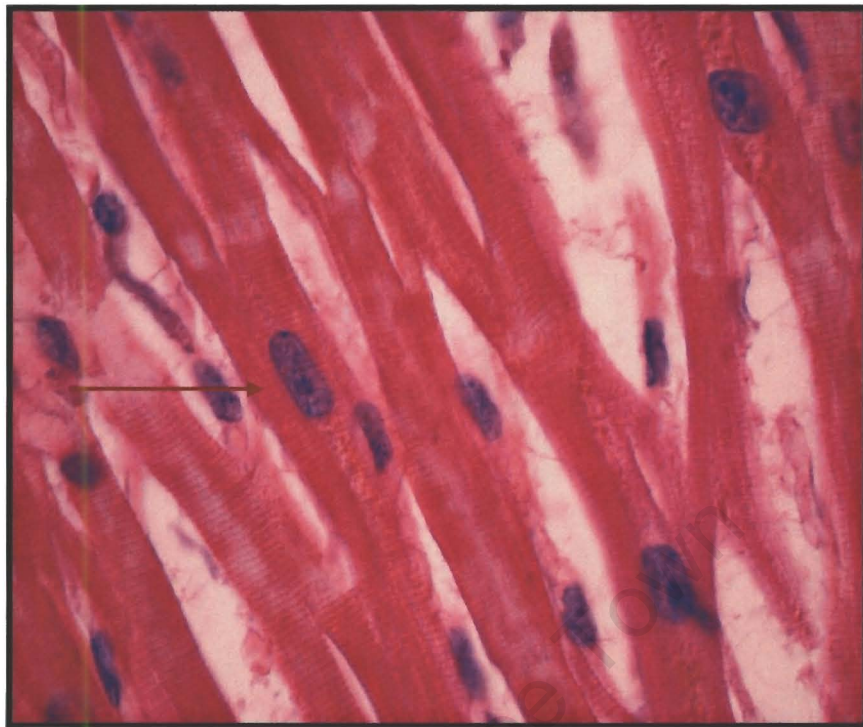
Figure 3. A bar graph summarizing the distribution of the NYHA classification for the three cohorts

A relatively low average serum CD4 count of 246 cells per cubic millimeter (cells/mm²) was found in the HIV-associated cardiomyopathy patients; the normal range of CD4 cell count is 600 - 1000 cells/mm² in our laboratory. The CD4 count correlated inversely with viral load; no significant association though was found between these two variables (p=0.23).

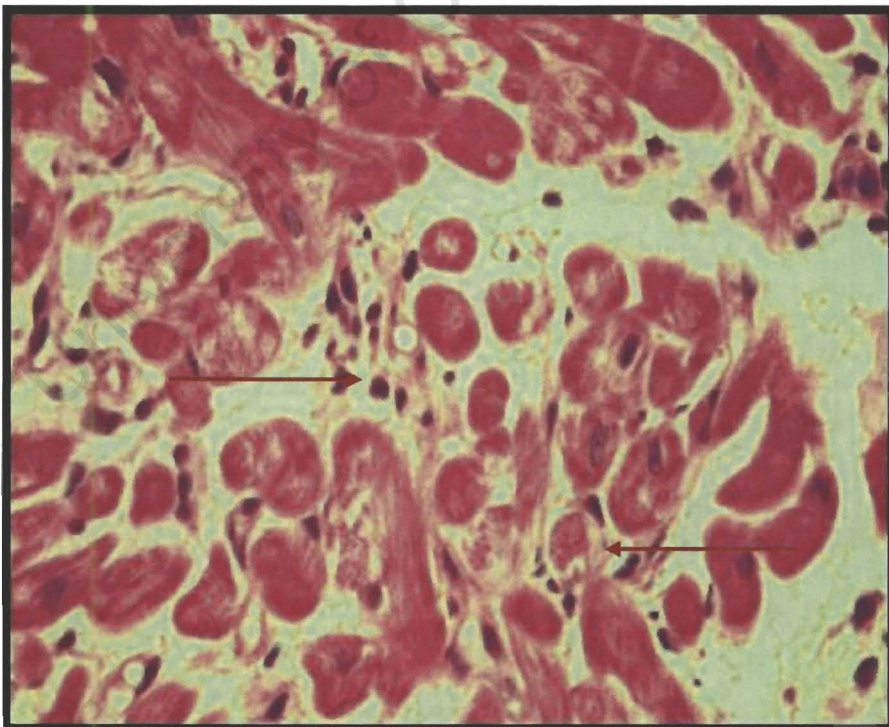
3.3.2 Prevalence of Myocarditis

We used light microscopy to obtain evidence of myocarditis in the endomyocardial biopsy tissues. Figure 4 shows the characteristics of the tissues investigated at the light microscopy level. As the current diagnostic criteria are Dallas and Marburg, our data are presented using both classification systems.

A.



B.



C.

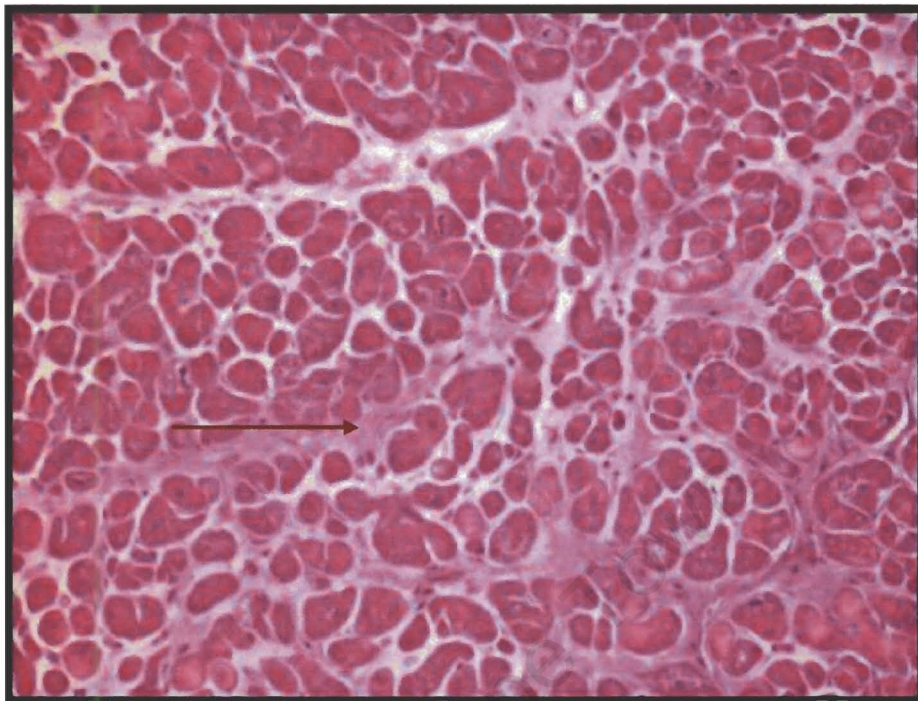


Figure 4. Light micrograph of **A.** An endomyocardial biopsy taken from the right ventricle of a rat. Note the normal distribution and appearance of the myocytes in the myocardium **B.** an endomyocardial biopsy of a heart transplant recipient showing a typical lesion of myocarditis in the right ventricle. Note the diffuse distribution of the lymphocytic infiltrate as well myocytolysis **C.** an endomyocardial biopsy taken from the right ventricle of a patient with HIVAC. Note the severe distribution of interstitial fibrosis throughout the tissue section; the fibrosis appears to be isolating various functional looking myocytes (hematoxylin-eosin stain; magnification x40).

3.3.3 Prevalence of Myocarditis by Dallas Criteria

3.3.3.1 HIV-associated cardiomyopathy

According to the Dallas criteria, 10/14 (71%) of patients with HIV-associated cardiomyopathy had histological features of myocarditis. Active myocarditis (i.e., lymphocytic infiltrate with myocytolysis) occurred in 4/14 (28.6%) cases and borderline myocarditis (i.e., lymphocytic infiltration with no myocytolysis) in 6/14 (42.8%) of the cases with HIV-

associated cardiomyopathy. No evidence of myocarditis was seen in 4/14 (28.6%) of HIV-associated cardiomyopathy cases (Figure 5).

Fibrosis was found in 10/14 (71%) of HIV-associated cardiomyopathy cases, which was mild in all except 3 cases which showed extensive myocardial fibrosis. Eight of the fourteen (50%) HIV-associated cardiomyopathy cases that displayed fibrosis at the light microscopy level also had evidence of myocarditis.

3.3.3.2 Heart transplant recipients

In this cohort we found that all cases 11/11 (100%) showed features of myocarditis (myocytolysis and/or inflammation). Of the heart transplant recipient cases 4/11 (36.3%) had active myocarditis and 7/11 (63.6%) were diagnosed with borderline myocarditis; none of the patients were free from myocardial inflammation (Figure 5). The entire heart transplant recipients cohort 11/11 (100%) had evidence of interstitial fibrosis.

3.3.3.3 Idiopathic dilated cardiomyopathy

None of the idiopathic dilated cardiomyopathy cases met the Dallas criteria for active/acute myocarditis; only mild to moderate lymphocytic infiltration and no myocytolysis was seen on histology. All eight were diagnosed as having borderline myocarditis 8/8 (100%), which was confirmed by immunohistochemistry (Figure 5). All of the idiopathic dilated cardiomyopathy cases 8/8 (100%) displayed interstitial fibrosis at the light microscopy level.

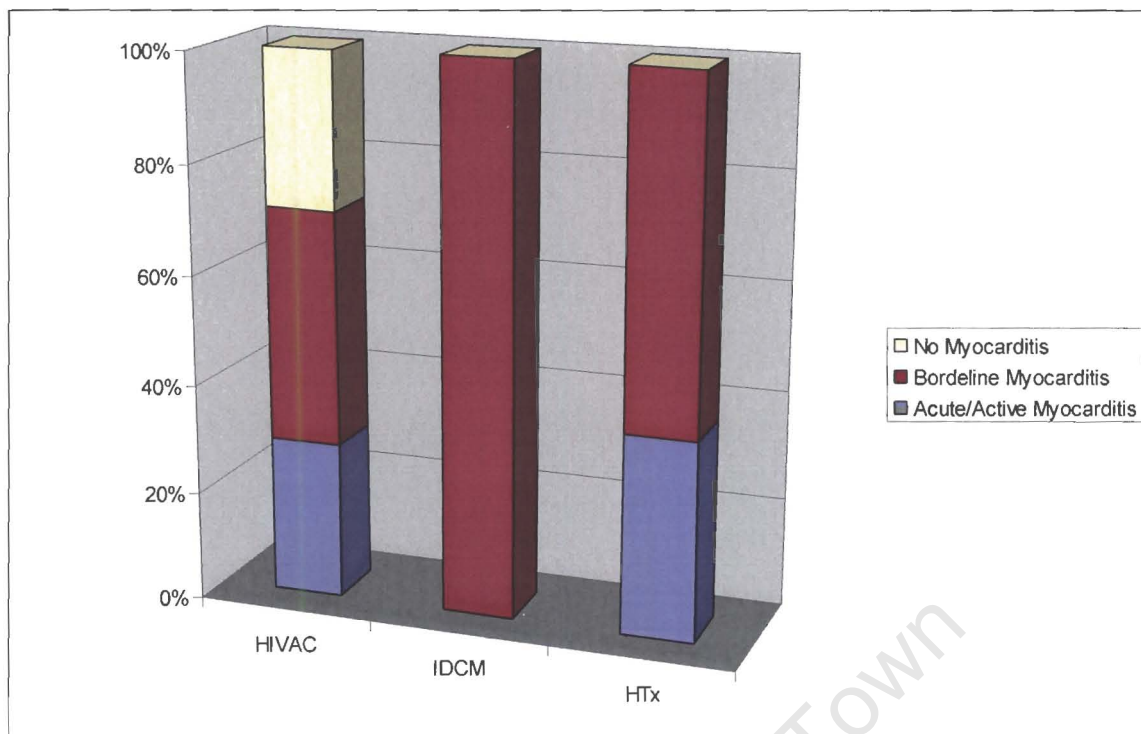


Figure 5. Prevalence of myocarditis by Dallas criteria in patients with HIV-associated cardiomyopathy (HIVAC), idiopathic dilated cardiomyopathy (IDCM), and heart transplant recipients (HTx).

3.3.4 Prevalence of Myocarditis by Marburg Criteria

Using the CD3 immune cell counts, we determined that 6/14 (42.8%) of the HIV-associated cardiomyopathy cohort met the Marburg inclusion criteria for myocarditis displaying ≥ 14 leukocytes/mm²; three cases had acute myocarditis 3/14 (21.4%), while three had chronic myocarditis 3/14 (21.4%). For the heart transplant recipients cohort, 4/11 (36%) of cases met the Marburg criteria for myocarditis; two cases had acute myocarditis 2/11 (18%), while the other two had chronic myocarditis 2/11 (18%). Two of the eight 2/8 (25%) idiopathic dilated cardiomyopathy cases met the Marburg criteria for chronic myocarditis (Figure 6).

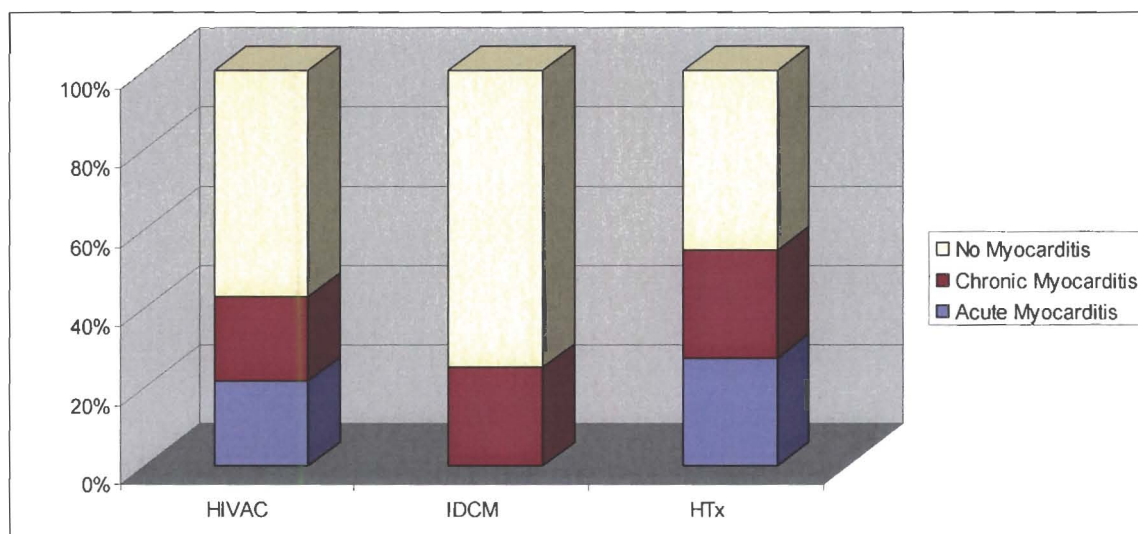


Figure 6. Prevalence of myocarditis by Marburg criteria in patients with HIV-associated cardiomyopathy (HIVAC), idiopathic dilated cardiomyopathy (IDCM), and heart transplant recipients (HTx).

3.3.5 Quantitation and characteristics of the inflammatory cell infiltrates in cases and controls

Immunophenotypic analysis by immunohistochemistry of the mononuclear infiltrates in the myocardium demonstrated a lymphocyte population expressing the pan T cell marker CD3, CD4⁺ T cells (helper/inducer), CD8⁺ T cells (cytotoxic/suppressor), and monocytes (CD68⁺ cells) (Figure 7).

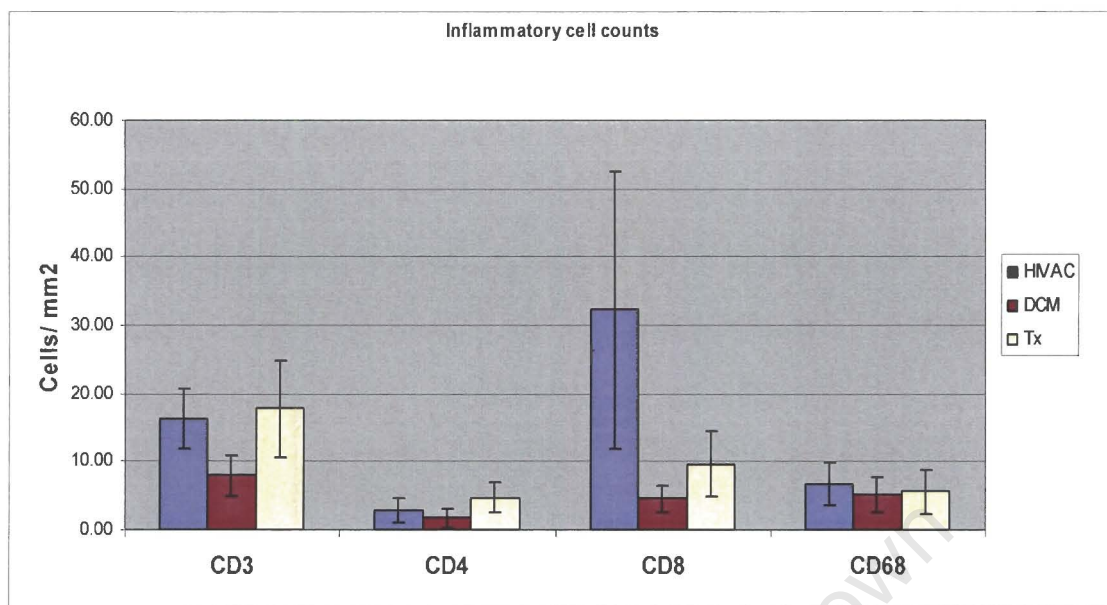


Figure 7. Bar graph comparing the immune cell populations in patients with HIV-associated cardiomyopathy (HIVAC), idiopathic dilated cardiomyopathy (DCM), and heart transplant recipients (TX).

CD8⁺ immune cells were the most prominent cell populations in HIV-associated cardiomyopathy (32.3 ± 20.3 cells/mm²), compared to heart transplant recipients (9.7 ± 4.9 cells/mm²) and idiopathic dilated cardiomyopathy (4.69 ± 1.9 cells/mm²). The average CD3⁺ cell count in HIV-associated cardiomyopathy of 16.39 ± 4.3 cells/mm² was similar to heart transplant recipients (17.89 ± 7.1 cells/mm²), but higher than idiopathic dilated cardiomyopathy cases (7.39 ± 3.0 cells/mm²). The prevalence of CD4⁺ and CD68⁺ cells was similar in all three groups (Table 6). The p-value ($p=0.107$) indicates that no statistically significant association was found between the myocytolysis positive and myocytolysis negative groups of all three groups.

Table 6 Summary of immune cell counts in the myocardium

Cohorts	CD3 tissue count		p-value	CD8 tissue count		p-value
	(cells/mm2)			(cemms/mm2)		
	Mean	SEM		Mean	SEM	
HIVAC	16.29	4.37		32.26	20.26	
Transplants	17.78	7.10		9.69	4.89	
IDCM	7.93	3.0		4.56	1.92	
Myocytolysis + vs myocytolysis -			0.046			0.107

In order to explain the differences in lymphocyte count between the groups, we examined the amount of inflammation (using immune cells CD3⁺ and CD8⁺) in patients with and without myocytolysis, a marker of acute/active myocarditis by both the Dallas and Marburg criteria. The data indicated that the groups with higher CD3⁺ immune cell counts (i.e., the pan T cell marker) were more than likely to be associated with myocytolysis than the groups that did not have myocytolysis (p=0.046)

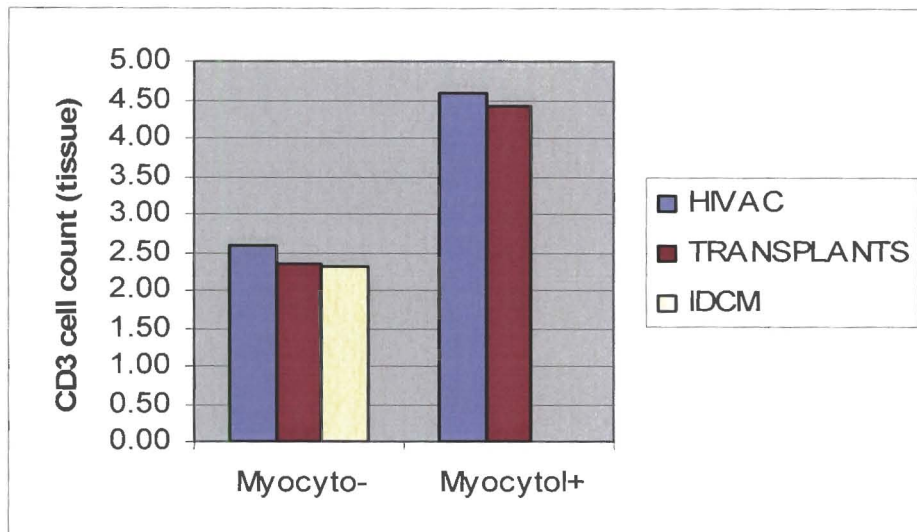


Figure 8 Summary of myocytolysis results (a marker of acute/active myocarditis) and CD3+ cell count in HIV-associated cardiomyopathy (HIVAC), idiopathic dilated cardiomyopathy (IDCM), and the heart transplant recipient (TRANSPLANTS) cohorts. Patients with active/acute myocarditis (i.e., myocytolysis present) had a significantly greater infiltrate of CD3+ cells (or lymphocytic infiltrate) than those without active/acute myocarditis.

Acute/active myocarditis was not found in patients with idiopathic dilated cardiomyopathy, which probably explains the lower levels of CD3+ cells in this group (Table 6; Figure 8).

No significant association ($p=0.107$) was found between CD8+ cell counts and myocytolysis (Figure 9). Between group analysis for CD3+ and CD8+ cell counts and myocytolysis was not done as values of 0 made statistical analysis difficult.

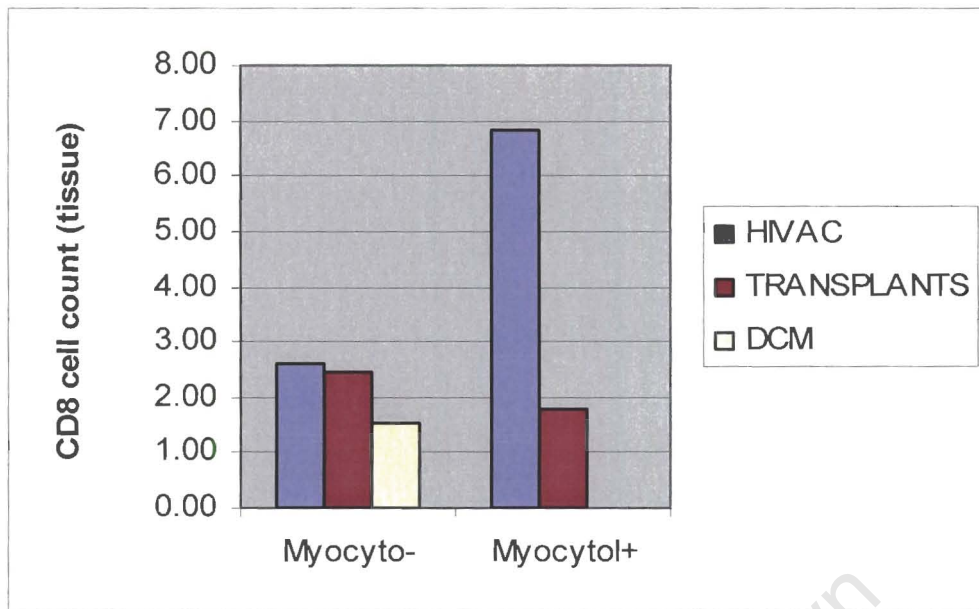


Figure 9. Summary of myocytolysis results and CD8 cell counts in idiopathic dilated cardiomyopathy (IDCM), HIV-associated cardiomyopathy (HIVAC) and the heart transplant recipient (TRANSPLANT) cohorts. The p-value was not significant.

3.3.6 Survival status

A Kaplan-Meier graph was constructed to determine if any difference in survival existed between the groups over a six month follow-up period (Figure 10).

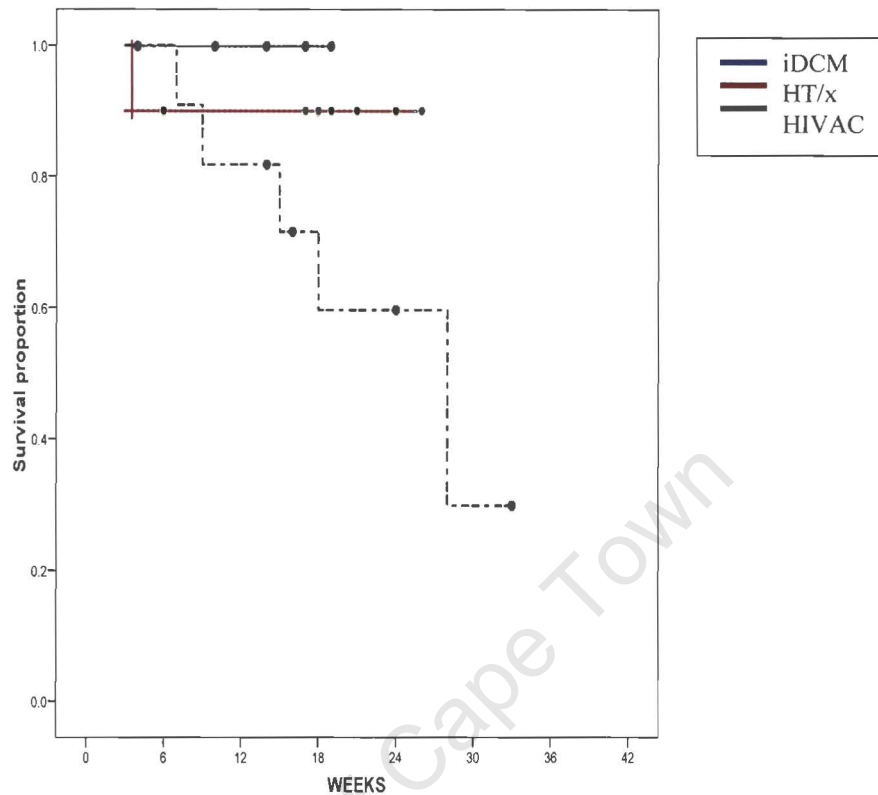


Figure 10. Kaplan-Meier graph depicting survival in the three groups 6 months post endomyocardial biopsy.

In the HIV-associated cardiomyopathy cohort 9/12 patients (71%) were dead at 6 months of follow-up; two HIV-associated cardiomyopathy patients had been lost due to follow-up. No deaths occurred in the idiopathic dilated cardiomyopathy group. Only one death occurred over six months in the heart transplant recipients group. We were unable to ascertain an outcome for two cases in the idiopathic dilated cardiomyopathy group as they were lost due to follow up.

The presence of myocarditis did not influence the risk of death in any of the groups. The log-rank test gave a value of $p=0.30$, indicating that the median survival time did not differ significantly in the three groups despite the apparent difference of the HIV-associated cardiomyopathy group compared to the other two cohorts. The incidence

of death in the HIV-associated cardiomyopathy group calculated to be 123.06 per 100 patients per year, 32.82 per 100 heart transplant recipients patients per year and 0.00 patients per year in the idiopathic dilated cardiomyopathy cohort.

3.3.7 Morphological abnormalities in cardiac myocytes

In addition to the mononuclear inflammatory cell infiltrates, myocytolysis and interstitial fibrosis described above, the following abnormal histological changes were also observed in all patient groups: reactive changes in myocyte nuclei and nucleomegaly (a histological marker of myocardial hypertrophy), prominent nucleoli and large vesicular nuclei (Figure 11); the arrow in this figure pinpoints a vacuole.

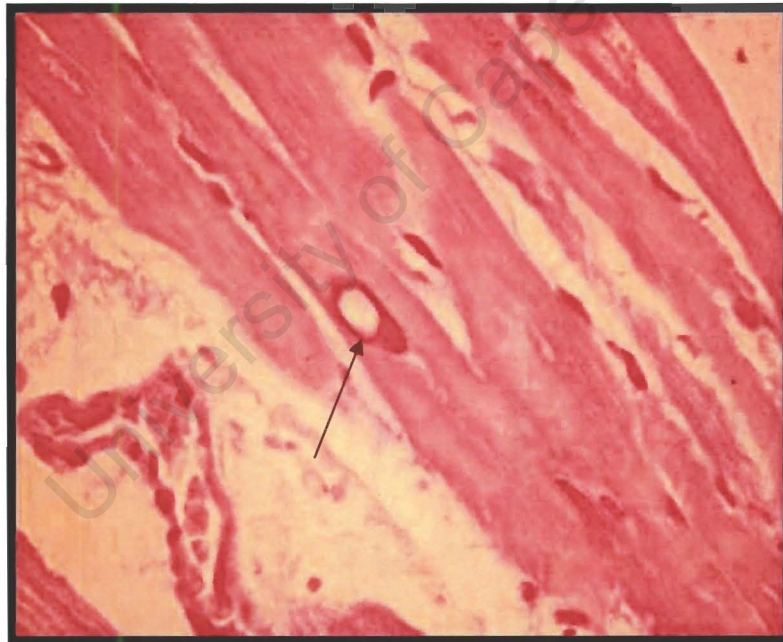


Figure 11. Other morphological changes observed. A light micrograph of an endomyocardial biopsy taken from the right ventricle of a HIV-associated cardiomyopathy patient. Note the vacuoles indenting the nucleus contain prominent intracytoplasmic vacuoles (hematoxylin-eosin stain; magnification x40)

All HIV-associated cardiomyopathy cases displayed nucleomegaly, 36% had myocyte vacuoles, while 92% showed irregular nuclear envelopes and only 50% showed nuclear blebs, the presence of nucleoli and myocyte vacuoles. Hypochromasia was present in 33% of the HIV-associated cardiomyopathy cases.

All idiopathic DCM cases displayed nucleomegaly and an irregular nuclear envelope while 50% showed prominent nucleoli and nuclear blebs; 38% showed the presence of myocyte vacuoles.

In the heart transplant recipients, the most prominent histological findings were nucleomegaly (91%), irregular envelopes (73%), followed by nucleoli (55%) and nuclear blebs (45%); only 9% displayed myocyte vacuoles. Vasculitis, granulomas and apoptotic myocytes were not observed.

Table 7 summarizes the differences and or similarities (as a percentage) between the histological findings of the groups studied at light microscopy level. No statistical significance was associated with any of the histological changes.

Table 7 Summary of the histomorphological changes (either than myocarditis) in the three groups of patients at light microscopy

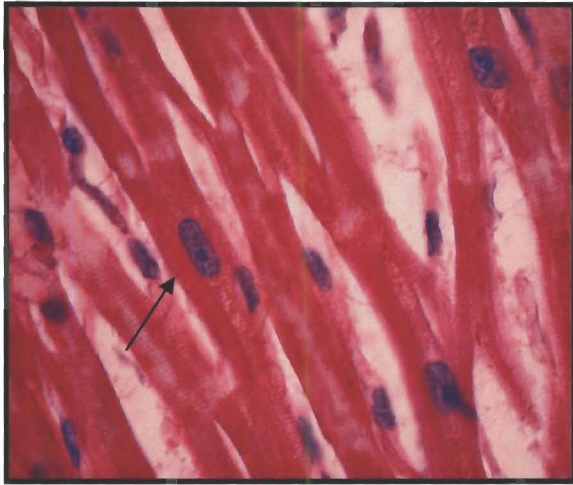
	HIVAC (%)	Heart transplant (%)	Idiopathic DCM (%)	p-value
Cytoplasmic vacuoles	36	9	38	0.09*
Nuclear membrane blebs	64	45	50	1.0*
Irregular nuclear envelope	93	73	100	0.18
Nucleomegaly	100	91	100	0.6*
Nuclear squaring	0	18	13	0.34*
Prominent nucleoli	43	55	50	1.0*
Nuclear hyperchromasia	0	0	13	0.26*
Nuclear hypochromasia	22	18	13	0.65*

*-Fisher's Exact Test used

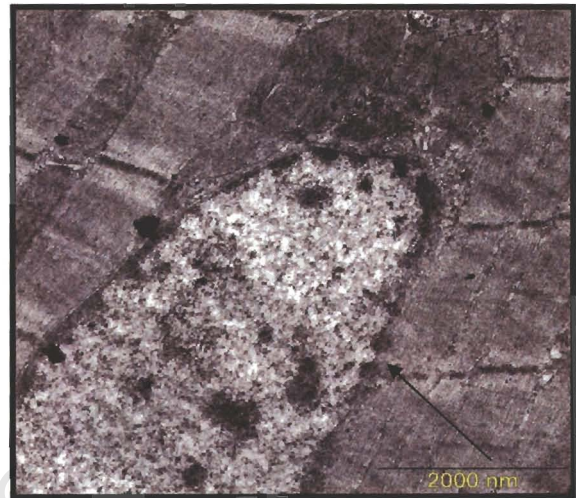
3.3.8 Electron Microscopy

Electron microscopy confirmed the nuclear changes seen at the light microscopy level as well as the presence of cytoplasmic vacuoles (Figure 12). The large, misshapen nuclei as well as the elaborate folding patterns of the nuclear lamina are clearly apparent. These nuclear abnormalities point to structural defects in the nuclear lamina.

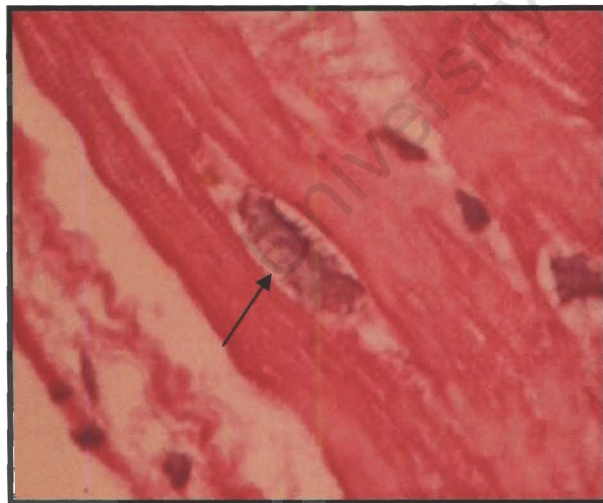
A



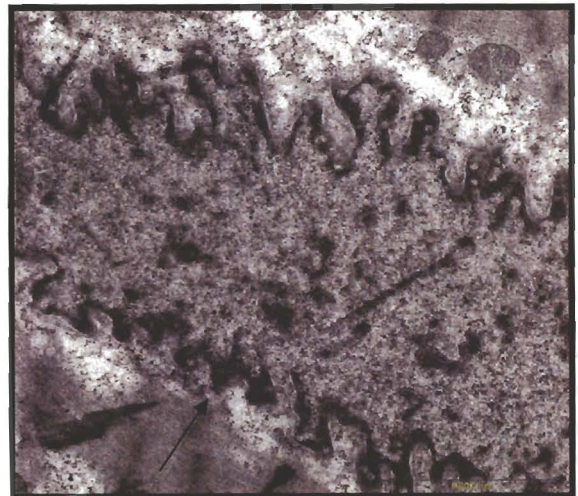
B.



C.



D.



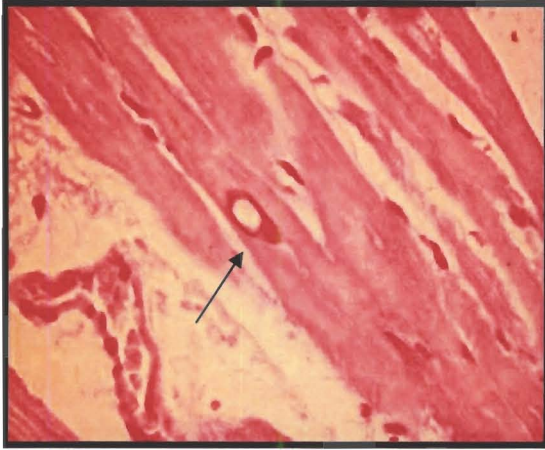
E.**F.**

Figure 12. Nuclear changes observed A. a light micrograph of an endomyocardial biopsy taken from the right ventricle of a rat (hematoxylin-eosin stain; magnification x40). Observe the normally shaped nuclei B. an electron micrograph of the same tissue showing the normal nuclear envelope C. a light micrograph on an endomyocardial biopsy taken from the right ventricle of a HIV-associated cardiomyopathy patient, note the abnormally shaped nucleus with the frilly projections (hematoxylin-eosin stain; magnification x40) D. an electron micrograph of the same tissue showing the abnormal folding of the nuclear membrane E. a light micrograph of cytoplasmic vacuoles F. an electron micrograph of an endomyocardial biopsy taken from the right ventricle of a patient, note the vacuoles filled with water.

No viral particles were evident in any of the three groups of patients investigated.

3.3.9 Fluorescence Microscopy

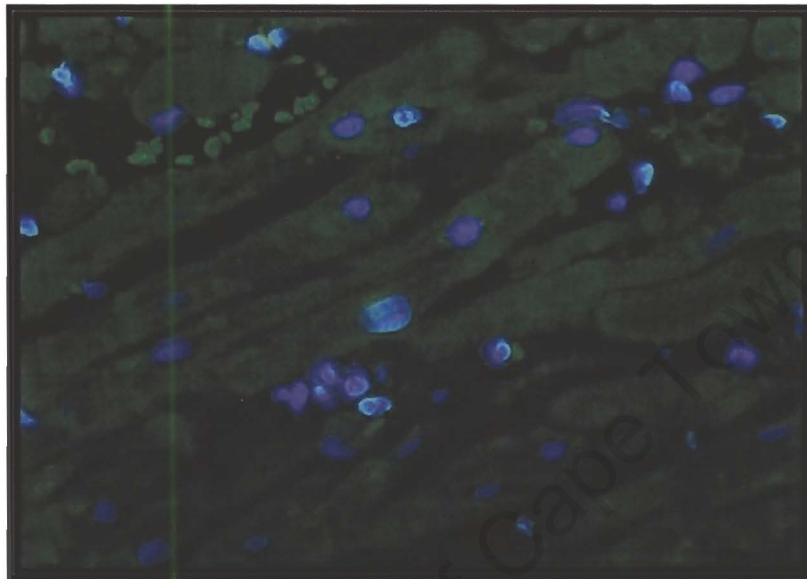
To account for the nuclear defects present we examined nuclear envelope markers in these cardiac tissues.

In a pilot study, we investigated the nuclei of cardiac myocytes through immunofluorescence and found evidence of chromatin

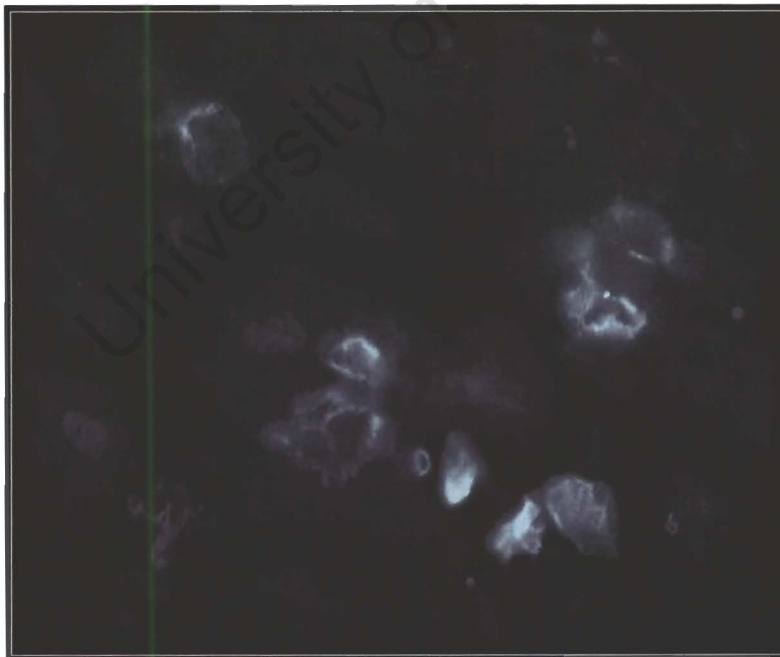
herniations, irregularities in nuclear lamina and disarray of nuclear pore complexes and compared these with normal control samples.

Fluorescent microscopy images (Figure 13) revealed small defects in lamin B1 signal in the nuclear lamina of an HIV-associated cardiomyopathy patient; this is contrasted against a relatively normal appearing nuclear lamina from the same section.

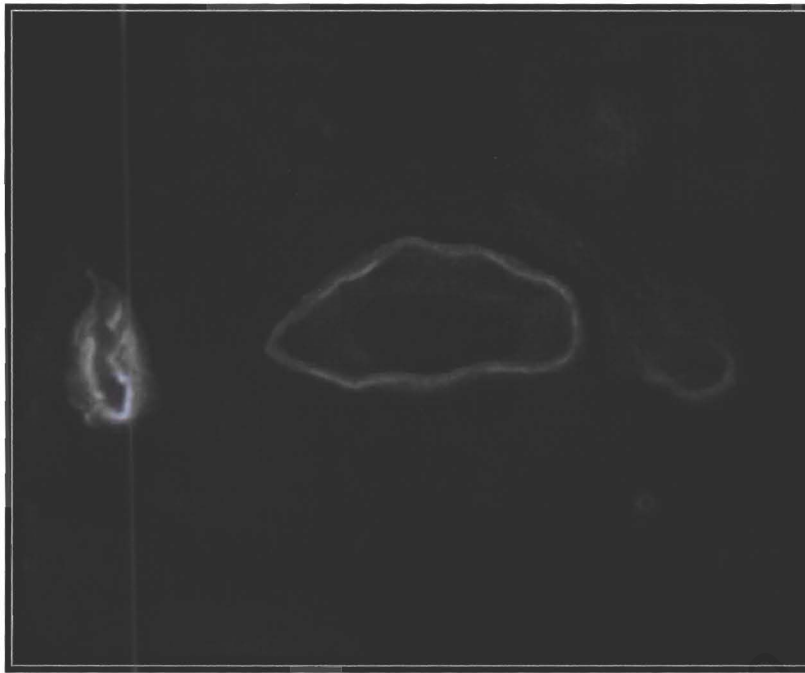
A.



B.



C.



D.

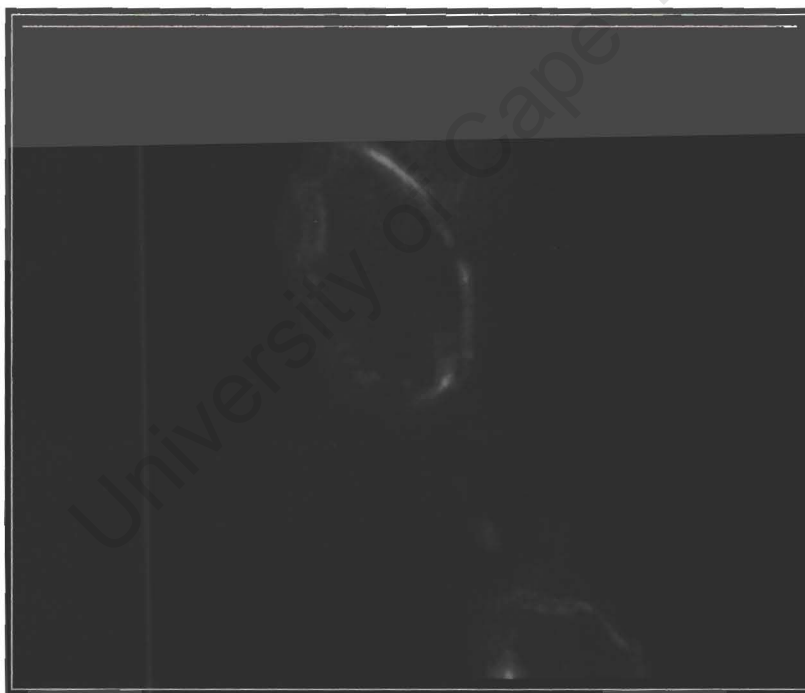


Figure 13. Fluorescent microscopy image of **A.** normal appearing myocytes **B.** nuclear lamina with nuclear lamin antibody B1, notice the nuclear herniations of the nuclear lamina **C.** the nuclear lamin B1 antibody in HIV-associated cardiomyopathy, notice the normal appearing lamina in a normal myocyte **D.** nuclear lamin B1 antibody, notice the interrupted lamina in an HIV-associated cardiomyopathy myocyte.

3.4 DISCUSSION

3.4.1 Myocarditis

To the best of our knowledge we report the first comprehensive endomyocardial biopsy study of HIV-associated cardiomyopathy in Africa, the epicenter of the global HIV pandemic. The application of both the Dallas and Marburg criteria indicate that acute/active myocarditis affects a minority of patients with HIV-associated cardiomyopathy, occurring in 21.4% (Marburg) to 28.6% (Dallas) of patients. The vast majority of patients have either borderline myocarditis (42.8%) or chronic myocarditis (21.4%), or no evidence of myocarditis at all.

The main finding in the autopsy studies was that of active myocarditis as the underlying pathological abnormality of HIV-associated cardiomyopathy. Consensus though, was not reached by all of these studies, as quite a number did not observe myocarditis. Controversy surrounded the diagnosis of myocarditis and the cause for the debate may be a result of a number of contributing factors, including (1) lack of uniformity in laboratory techniques used to identify pathogens; some studies only used light microscopy, or viral cultures but no immunohistochemistry, (2) the use of different diagnostic criteria of myocarditis; some studies use the Dallas criteria to identify myocarditis while others did not mentioned their criteria at all or some even modified the criteria, (3) differences in the stage of disease or treatment of patients infected with HIV (e.g., some studies reported that patients had been on antiretroviral therapy whereas other patients had not) and, lastly (4) whether autopsy tissue (likely to be associated with late disease) or endomyocardial biopsy specimens (likely to be associated with early disease) are used.

Like the other studies on HIV-associated cardiomyopathy, we used light microscopy, electron microscopy and immunohistochemistry to characterise the tissues at the structural level. However, unlike previous

studies, we applied the two grading systems for the diagnosis of myocarditis which are qualitative (i.e., the Dallas criteria) and quantitative (i.e., Marburg criteria), and thus complimentary in the diagnosis of the myocarditis. Indeed, this is probably the first study to apply the Marburg criteria to diagnose myocarditis in HIV-associated cardiomyopathy to date.

Immunohistochemistry showed that the lymphocytic infiltrate seen in the myocardium of the HIV-associated cardiomyopathy patients was mainly CD8⁺ immune cells. The fact that there were more CD8⁺ cells than other cell types in the HIV-associated cardiomyopathy myocardium is not surprising as CD8⁺ cells are cytotoxic T cells that are responsible for destroying virus infected cells; this finding was suggestive of a viral infection (whether latent or active infection is uncertain).

3.4.2 Other histological findings

In the three cohorts, fibrosis was a common finding at the light microscopy level. The fibrosis seen could be rationalized by one of a few scenarios. The fibrosis seen in all three cohorts could be the result of a healed myocarditis (according to the Marburg criteria) or a resolving myocarditis (according to the Dallas criteria). The fibrosis could also be the result of a chronic low-grade infection (not active myocarditis) caused by reactivation of cardiotropic viruses; this low grade infection could result in a chronic release of circulating inflammatory factors that damage the myocytes and result in fibrosis and a failing heart.

Also significant is the fact that we have found the presence of cytoplasmic vacuoles in the myocytes of patients with HIV-associated cardiomyopathy and idiopathic dilated cardiomyopathy. Literature has reported the presence of these vacuoles in myocytes undergoing degeneration (Turillazzi *et al* 2005, Owens *et al* 2006). Special stains on these tissues (Bests Carmine stain) revealed that the vacuoles did not contain glycogen or lipid (electron microscopy); based on these findings

we believe that the vacuoles were filled with water. Electron microscopy demonstrated myocytes undergoing hydropic degeneration.

Due to the fact that all three groups displayed nuclear abnormalities (such as nucleomegaly and irregular nuclear envelope) and that all groups displayed inflammation, it may be possible that these nuclear abnormalities are markers of cellular degeneration. Similar nuclear envelope changes to ours have been reported in hereditary forms of cardiomyopathy where it was thought that defects in the genes encoding nuclear membrane proteins caused the heart disease (Hershberger 2002, Fatkin 1999, Bergo 2004).

The nuclear lamina forms a scaffold for the nuclear envelope to attach, for nuclear pore complexes to be anchored, and for organisation of the peripheral nuclear chromatin and DNA. The nuclear lamina may be crucial for nuclear organisation and gene regulation (Hutchison 2002). The nuclear lamina is comprised of the lamin proteins, of which there are highly homologous A-type and B-type isoforms. The A-type lamins are expressed in terminally differentiated cells and have been implicated in a variety of inherited clinical syndromes including cardiomyopathies, muscular dystrophies, lipodystrophy and Hutchinson-Gilford progeria syndrome (an inherited form of premature ageing) (De Sandre-Giovannoli 2003; Goldman 2002). The B-type lamins are essential developmental proteins and are ubiquitously expressed. In addition, they are modified by post-translational modifications at the carboxyl terminus - the so called CAAX-dependent modifications. These modifications involve sequential addition of a lipid group to the protein, followed by removal of a small part of the protein (endoproteolysis) and finally methylation of the carboxyl terminus of the protein to produce a hydrophobic end that can insert into the nuclear envelope (Maske 2003). However it is thought that more subtle changes to protein function through aberrant protein modification and posttranslational modification by cellular

enzymes such as Rce1, ICMT and farnesyl transferase may result in acquired loss of function.

The hypothesis arising from this study therefore predicts that acquired changes in Rce1 activity in cardiac myocytes results in aberrant processing of lamin B1 and that this results in a cardiomyopathy similar to inherited cardiomyopathies involving lamin A mutations (Fatkin 1999, Bergo 2004). In the case of HIV-positive individuals, acquired changes to Rce1 activity may be the result of cell stress due to viral infection, responses to immune mediators or direct inhibition by anti-retroviral drugs.

We speculate that the molecular mechanism by which the nuclear envelope morphology is altered in inflammatory cardiomyopathy is similar to that seen in inherited forms of cardiomyopathy, where circulating inflammatory factors might cause mechanical stress and cell death.

3.4.3 Survival

The study of outcome in HIV-associated cardiomyopathy is to the best of my knowledge the first of its kind in Africa. The patients with HIV-associated cardiomyopathy had an extremely poor prognosis, compared to patients with idiopathic dilated cardiomyopathy and heart transplant recipients. The incidence of death in the HIV-associated cardiomyopathy group was 123 per 100 patients per year, 33 per 100 heart transplant recipients patients per year and no deaths were predicted in those with the idiopathic dilated cardiomyopathy. These results confirm the findings of other investigators, that HIV-associated cardiomyopathy, in the absence of anti-retroviral treatment, is associated with a dismal prognosis (Felker 2000).

3.5 CONCLUSION

This study contributes to new knowledge in at least four ways. First, we show that active/acute myocarditis accounts for a minority of patients with HIV-associated cardiomyopathy (approximately 25%) in Africans. This is in contrast to others who have suggested that active/acute myocarditis is the pathological hallmark of HIV-associated cardiomyopathy (Longo-Mbenza 1998, Barbaro 2001). Second, there is a marked increase in CD8+ lymphocytes in the heart tissue of patients with HIV-associated cardiomyopathy. This response may be indicative of either direct or indirect viral infection of the cardiac muscle. The role of viral infection in HIV-associated cardiomyopathy is examined in Chapter 4 of this thesis. Third, we confirm that HIV-associated cardiomyopathy is associated with an extremely poor prognosis without treatment with anti-retroviral drugs. Therefore, the treatment of HIV-associated cardiomyopathy is not anti-heart failure treatment, but anti-retroviral drugs. Finally, based on the nuclear changes found in the biopsy tissues of all three groups with varying degrees of myocardial inflammation, we postulate that inflammatory cardiomyopathy may be characterised by an acquired form of a nuclear laminopathy. This hypothesis requires to be tested in future studies.

CHAPTER 4

DETECTION OF VIRAL GENOMES IN THE MYOCARDIUM OF PATIENTS WITH HIV- ASSOCIATED CARDIOMYOPATHY

CHAPTER 4: DETECTION OF VIRAL GENOME IN THE MYOCARDIUM OF PATIENTS WITH HIV-ASSOCIATED CARDIOMYOPATHY

4.1 INTRODUCTION

The fact that viral genomes are frequently detected in human hearts with idiopathic dilated cardiomyopathy suggests that viral infections may play a role in the pathogenesis of idiopathic dilated cardiomyopathy. But the definitive diagnosis of viral myocarditis has remained elusive with positive viral cultures from the heart rarely obtained (Martin 1994).

Techniques, such as serology, viral culture, immunohistochemistry and histopathology have sought to diagnose viral myocarditis in a sensitive and accurate way (Martin 1994). Unfortunately, cultures obtained from myocardial tissue in patients with idiopathic dilated cardiomyopathy or histology-proven myocarditis was usually negative for viral infection. Immunohistochemical studies often fail to detect viral antigens from these specimens, and it has been speculated that this reflects either the focal nature of the viral myocardial infection, sampling bias, the small number of viral particles present in chronic infections, or clearing of the virus by a competent immune system (Griffin 1995). Studies by Chow (1989) and Hauck (1989) independently found that the probability of diagnosing myocarditis by light microscopy using four to five biopsy samples was approximately 50%; the highest sensitivity achieved was 79% with 17 biopsy samples obtained. Most of these studies were time consuming though, generally falling short in specificity and sensitivity and appearing to definitively identify only a proportion of patients with myocarditis. Additional diagnostic methods were needed to diagnose acute viral myocarditis with improved diagnostic accuracy (Bowles 1986, Bowles 1989).

The introduction of the polymerase chain reaction though, made possible the direct detection of viral genomes in myocardial tissue of patients with idiopathic dilated cardiomyopathy or myocarditis.

A summary of the prevalence rates for cardiotropic viruses in the myocardium of HIV-associated cardiomyopathy, myocarditis and idiopathic dilated cardiomyopathy is presented in Table 8.

In chapter 3, we found that patients with HIV associated cardiomyopathy exhibited the full spectrum of inflammatory myocardial disease, with a quarter of patients having active/acute myocarditis, about half with borderline/chronic myocarditis, and the remainder with no evidence of myocardial inflammation. Furthermore, there was a predominance of myocardial CD8+ lymphocytes in HIV-associated cardiomyopathy, suggesting that this may be a response to direct viral infection of the myocardium, either by HIV itself and/or by other cardiotropic viruses. We sought, in this chapter, to establish the role of viral infection in the pathogenesis of HIV-associated cardiomyopathy by comparing the presence of myocardial viral genomes with patients with idiopathic dilated cardiomyopathy and heart transplant recipients. To achieve this aim, DNA and RNA was extracted from the endomyocardial biopsies of these patients.

Table 8 Cardiotropic viruses and their prevalence in cardiac tissue

	Prevalence (%)	Cohort description	Reference
<u>Overall viral genomes in cohorts</u>			
HIVAC	31-42	HIV-1 cardiomyopathy	Grody 1990, Herskowitz 1994, Barbaro 1998, Bowles 1999, Barbaro and Lipschultz 2001, Barbaro 2001, D'Amanti 2001, Currie and Boon 2003, Barbaro 2005
Transplant recipients	20-70	Myocarditis	Martin 1994, Baboonian and Treasure 1997, Barbaro 1998, Pauschinger 1999, Bowles 1999, Bowles 2003
Idiopathic DCM	20-70	DCM	Martin 1994, Griffin 1995, Baboonian and Treasure 1997, Barbaro 1998, Bowles 1999,, Pankuweit 1998; Pankuweit 1999 1999, Pauschinger 1999, Mantke 2003, Baboonian 2003, Bowles 2003, Kuhl 2003, Kuhl 2005, Poller 2005, Pankuweit 2005
<u>Viruses</u>			
Enterovirus	3-36	Myocarditis/DCM	Martin 1994, Griffin 1995, Barbaro 1998, de Leew 1999, Pauschinger 1999, Klingel 2002, Bowles 2003, Kuhl 2003, Pankuweit 2005, Kuhl 2005, Poller 2005
Adenovirus	1.6-58	Myocarditis/DCM	Martin 1994, Griffin 1995, Pauschinger 1999, Bowles 1999, Klingel 2002, Bowles 2003, Kuhl 2003, Kuhl 2005, Poller 2005, Pankuweit 2005

	Prevalence (%)	Cohort description	Reference
<u>Viruses(cont.)</u>			
Parvovirus	7-50	Myocarditis/DCM	Pauschinger 1999, Klingel 2002, Kuhl 2003, Kuhl 2005, Poller 2005, Pankuweit 2005
CMV	3-48	Myocarditis/DCM	Martin 1994, Herskowitz 1994, Griffin 1995, Barbaro 1998, Pauschinger 1999, de Leew 1999, de Leew 1999, Klingel 2002, Bowles 1999, Currie and Boon 2003, Bowles 2003, Pankuweit 1998, Poller 2005
EBV	2-9.4	Myocarditis/DCM	Barbaro 2001, Pauschinger 1999, Kuhl 2003, Kuhl 2005, Poller 2005
HSV	3-17	Myocarditis/DCM	Martin 1994, Griffin 1995, Pauschinger 1999, Hogrefe 2002, Bowles 2003, Kuhl 2003, Kuhl 2005, Poller 2005
HIV-1	27-41	Myocarditis/DCM	Grody 1990, Herskowitz 1994, Barbaro 1998, Bowles 1999

4.2 METHODS

The selection of cases and controls, the endomyocardial biopsy techniques, and the methods for viral detection are described fully in chapter 2 (section 2.3).

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4.3 RESULTS

4.3.1 Number of endomyocardial biopsy samples used in this study

Fifteen patients with HIV-associated cardiomyopathy underwent endomyocardial biopsy for this study. One patient biopsy was excluded from the analysis due to tissue inadequacy (mainly adipose tissue, no myocardium) resulting in 14 patients being entered into the study. Fifteen heart transplant recipients' biopsies and ten idiopathic dilated cardiomyopathy patients were recruited and used as controls for comparison with findings in the patients with HIV-associated cardiomyopathy. Three heart transplant recipient biopsies and three idiopathic dilated cardiomyopathy were excluded from the study due to tissue inadequacy (i.e., adipose predominance, crushed section, biopsy too small), resulting in 12 and 7 patients being entered into the virology analysis, respectively.

4.3.2 Virology: Presence of viruses by reverse transcriptase polymerase chain reaction (RT-PCR) and nested-PCR (nPCR) analysis

All samples were positive for the in-house gene β -globin, by PCR and reverse transcriptase PCR, indicating the successful isolation of DNA and RNA from endomyocardial biopsies. A total of 33 DNA and RNA samples were PCR-tested for genomes of seven viruses (i.e., adenovirus, enterovirus, cytomegalovirus, herpes simplex virus, Epstein-Barr virus, parvovirus, and HIV-1) in the three cohorts (Figure 14).

A.



B.



C.

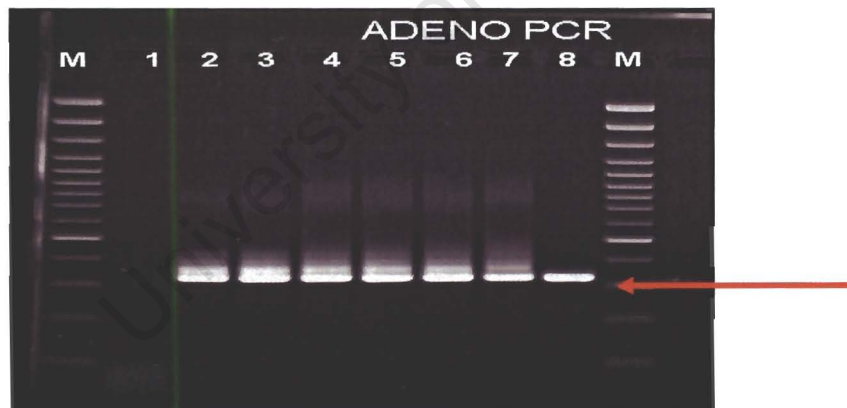




Figure 14 PCR results for the viruses studied **A.** a 1.5% agarose gel for the viruses CMV (160 base pairs), EBV (209base pairs) and HSV (179base pairs); six amplicons (each representing six patients) for each of the three viruses are shown respectively **B.** a 1.5% agarose gel showing the 397base pair amplicon for parvovirus **C.** a 1.5% agarose gel showing the 330base pair amplicon for adenovirus **D.** a 1.5% agarose gel showing the 155base pair amplicon for enterovirus. Arrows indicate bands of interest.

Viruses were amplified in the following proportions:

4.3.2.1 HIV-associated cardiomyopathy

Viral prevalence for HIV-associated cardiomyopathy cases was 100% as all 14 cases were infected with at least one virus by PCR. The number of viruses per case ranged from one ($n=4/14$; 29%) to four ($n=3/14$; 21%) with a mean viral burden of 2.3 viruses per case.

The biopsy tissues were dominated by the EBV and HSV viruses, with EBV occurring in 9/14 cases (64%) followed by HSV in 7/14 (50%) and enterovirus in 5/14 cases (38%). In addition, HIV-1 was detected in 4/14 (29%) HIV-associated cardiomyopathy cases. An explanation for this could be that not many of the circulating cells were infected with the HIV-1 virus.

4.3.2.2 Heart transplant recipients

A viral prevalence of 80% was found in the heart transplant recipient cases as 10/12 cases were positive for at least one virus by

PCR. The range of viruses was zero (n=2/12; 16%) to four (n=3/12; 25%) with a mean viral burden of 2.2 viruses per case.

The most prevalent virus was the enterovirus (7/12; 58%) followed by Adenovirus (6/12; 50%) and EBV (6/12; 50%).

4.3.2.3 Idiopathic dilated cardiomyopathy

A prevalence of 100% was found in the idiopathic dilated cardiomyopathy cases as all seven cases was found to be positive for at least one virus by PCR. The range of viruses were one (n=6/7; 86%) to two (n=1/7; 14%) with a mean viral burden of 1.1 viruses per case.

The most prevalent virus was the enterovirus (4/7; 57%) followed EBV, HSV, parvo and adenovirus at equal prevalence's of 1/7 (14%) respectively. No CMV was detected in this cohort (Table 9).

Table 9 Prevalence of viral genomes in the myocardium of patients with HIV-associated cardiomyopathy, heart transplant recipients and idiopathic dilated cardiomyopathy

	HIVAC (%)	HT/x (%)	IDCM (%)
Entero	5 (36)	7(58)	4(50)
Adeno	4(29)	6(50)	1(13)
Parvo	2(14)	2(17)	1(13)
CMV	1(7)	1(8)	0(0)
EBV	9(64)	6(50)	1(13)
HSV	7(50)	3(25)	1(13)
HIV	4(29)	0(0)	0(0)
sample total	14	12	8

A graph depicting viral presence in the myocardium of the three cohorts are presented in Figure 15.

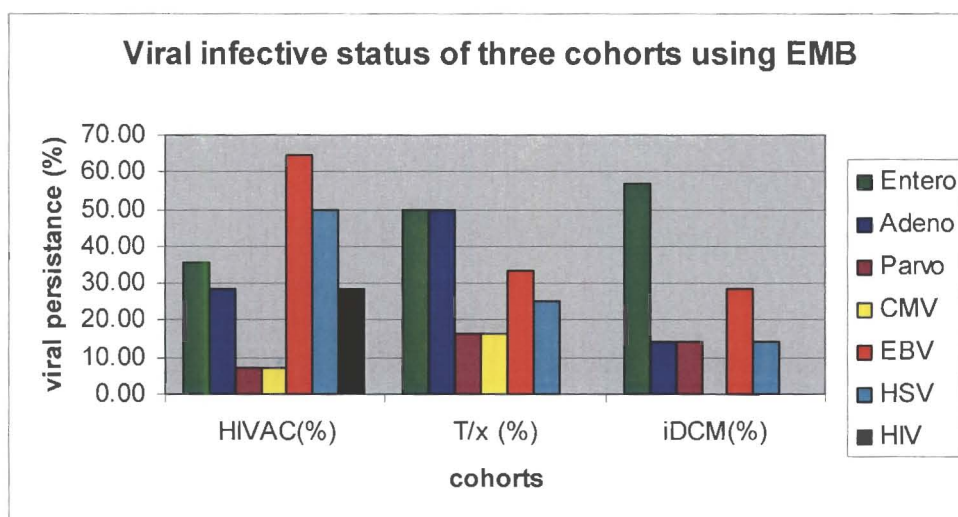


Figure 15 Graph of endomyocardial biopsy infectivity status of the three cohorts that were investigated in this study. Note the viral distributions in the various groups. EBV and HSV appear to be the most prevalent virus in HIV associated cardiomyopathy patients; with the entero-, adeno- and EBV viruses appearing with almost equal persistence in the transplanted tissue.

Viral prevalence rates for the three cohorts are presented in Table 12.

4.3.3 Viral Immunohistochemistry:

No viral presence was detected with any of the viral antibodies (Cytomegalo- , Epstein-Barr-, Herpes simplex-, Parvo-, Adeno-, Entero- and HIV-1 viruses) (Dak, Carpenteria; Novocastra, UK Laboratories) stained in any of the patient groups (i.e., HIVAC, idiopathic dilated cardiomyopathy, and heart transplant recipients). The control tissue (lymph nodes) stained positive for each virus respectively.

4.3.4 Cellular Immunohistochemistry (chapter3) and Virology comparison

In chapter 3, we found that patients with HIV-associated cardiomyopathy exhibited the full spectrum of inflammatory myocardial

disease. Furthermore, the predominance of myocardial CD8⁺ lymphocytes in this cohort suggested that this may be a response to direct viral infection of the myocardium, either by HIV itself and/or by other cardiotropic viruses.

A comparison of the immunohistochemistry results in chapter 3 and the virology (PCR) results in this current chapter was done in an attempt to answer this question.

The increase in the CD3⁺ (and the CD8⁺ subset) T cells in patients with active myocarditis (both HIV-associated cardiomyopathy and heart transplant recipients) confirms the pattern that was noted in chapter 3.

4.3.4.1 HIV-associated cardiomyopathy

Immunohistochemistry in chapter 3 had detected a high CD3⁺ cell count (31.07 cells/mm²) in the myocarditis positive group for this cohort; PCR detected an overall viral presence of 2.5 viruses per case. Immunohistochemistry also detected a high CD8⁺ cell count (83.01 cells/mm²) in the myocarditis positive group for this cohort; PCR detected an overall viral presence of 2.5 viruses per case.

We compared the degree of myocarditis (in groups with active myocarditis or no active myocarditis) with viral genome presence in the HIV-associated cardiomyopathy cohort and found that even though the CD8⁺ cell count was higher in active myocarditis group, there was no association with any one virus or group of viruses. There was also no obvious association between presence of viral genome and active myocarditis. However, the individuals with active myocarditis had a higher viral load than those without active myocarditis.

4.3.4.2 Heart Transplant recipients

Immunohistochemistry in chapter 3 detected a high CD3⁺ cell count (32.7 cells/mm²) for the myocarditis positive group in this cohort

and PCR detected an overall viral presence of 2 viruses per case.

Immunohistochemistry also detected a moderately high CD8⁺ cell count (15.5 cells/mm²) for the myocarditis positive group in this cohort and PCR detected an overall viral presence of 2.4 viruses per case.

We also compared the degree of myocarditis (in groups with active myocarditis or no active myocarditis) with viral genome persistence in the heart transplant recipient cohort; even though there was a high CD8⁺ cell count in the tissues of these patients with active myocarditis, there was no association with any one virus or group of viruses.

4.3.4.3 Idiopathic dilated cardiomyopathy

Immunohistochemistry (in chapter 3) detected no active myocarditis. A CD3⁺ cell count of 7.93 cells/mm² for the myocarditis negative group was found; PCR detected an overall viral presence of 1.1 viruses per case.

4.3.4.4 Statistical analysis

We performed a statistical analysis to determine if an association existed between a high CD3⁺ and/or CD8⁺ cell count and viral presence. We compared the mean CD3 and CD8 cell counts of each group within each virus. Statistical analysis found no association between any of the viruses present and the CD3⁺ and CD8⁺ cell counts for any of the cohorts tested.

A summary of the results obtained in chapter 3 and the current chapter (chapter 4) are presented in Tables 10-12. The clinical characteristics, the mononuclear cell infiltrate (in relation to the presence of active myocarditis) (chapter 3), and the prevalence of viral genomes in the different groups are presented (raw data available in Appendix 2).

Table 10. Summary of clinical characteristics, myocardial immune cell count, and prevalence of myocardial viral genome in HIV-associated cardiomyopathy, stratified by the presence or absence of active myocarditis (Dallas criteria)

		HIV associated cardiomyopathy	
		No active myocarditis	Active myocarditis
<u>Clinical Characteristics</u>			
Number (N)		10/14	4/14
Gender:	Male (%)	5/10 (50)	2/4 (50)
	Female (%)	5/10(50)	2/4(50)
Mean age ($\pm$ SD)		40 ($\pm$ 7.63)	35 ($\pm$ 7.63)
Mean LVEF ($\pm$ SD)		27 ($\pm$ 12)	29 ($\pm$ 8)
NYHA :			
	I	2/10	3 / 4
	II	4/10	0 / 4
	III	3/10	1/ 4
	IV	0/10	0/ 4
	Unknown	1/10	
Mean CD4 count (serum) ($\pm$ stdev)		293 ($\pm$ 236)	264($\pm$ 25)
MeanCD8 count (serum) ($\pm$ stdev)		784($\pm$ 278)	1409 ($\pm$ 844)
Mean Viral load copies/ml		660 337.4	1 541 000
<u>Immunohistochemistry (myocardium)</u>			
Mean CD3 cell count cell count /mm ² ($\pm$ stdev)		10($\pm$ 8.69)	31($\pm$ 22.8)
MeanCD4 cell count cell count /mm ² ($\pm$ stdev)		2($\pm$ 2.59)	7($\pm$ 11.5)
Mean CD 8 cell count /mm ² ($\pm$ stdev)		10($\pm$ 12.04)	83($\pm$ 156.9)
Mean CD 68 cell count /mm ² ($\pm$ stdev)		3($\pm$ 5.42)	19($\pm$ 18.74)
<u>Prevalence of viral genome by PCR</u>			
CMV (%)		1/10(10)	0/4 (0)
EBV (%)		6/10(60)	3/ 4 (75)
HSV (%)		5/10(50)	2/4 (50)
HIV-1 (%)		4/10(40)	0/4 (0)
Adenovirus (%)		2/10(20)	2/4 (50)
Parvovirus (%)		1/10(10)	1/4 (0)
Enterovirus (%)		3/10(30)	2/4 (50)
Mean viruses per case		2.2	2.5
Overall viral prevalence		100%	

Table 11 Summary of clinical characteristics, myocardial immune cell count, and prevalence of myocardial viral genome in heart transplant recipients, stratified by the presence or absence of active myocarditis (Dallas criteria)

		Heart transplant recipient cohort	
		No active myocarditis	Active myocarditis*
<u>Clinical Characteristics</u>			
Number (N)		8/ 12	4/ 12
Gender:	Male (%)	6/ 8 (75)	3/ 4 (75)
	Female (%)	2/ 8 (25)	1/ 4 (25)
Mean age ($\pm$ sd)		46 ($\pm$ 12.1)	48 ($\pm$ 17.6)
Mean LVEF ($\pm$ sd)		51 ($\pm$ 9.2)	56 ($\pm$ 4.7)
NYHA :	I	6/ 7 (100)	4/ 4 (100)
	II	0/ 7 (0)	0/ 4 (0)
	III	0/ 7 (0)	0/ 4 (0)
	IV	0/ 7 (0)	0/ 4 (0)
	Unknown	1/7	
<u>Immunohistochemistry (myocardium)</u>			
Mean CD3 cell count	cell count /mm ² ($\pm$ sd)	7.85($\pm$ 7.8)	32.7($\pm$ 32.7)
MeanCD4 cell count	cell count /mm ² ($\pm$ sd)	2.64($\pm$ 6.5)	8.8($\pm$ 8.1)
Mean CD 8 cell count	/mm ² ($\pm$ sd)	5.8($\pm$ 8.2)	15.5($\pm$ 24.6)
Mean CD 68 cell count	/mm ² ($\pm$ sd)	3.6($\pm$ 7.1)	9.62($\pm$ 16.7)
<u>Prevalence of viral genome by PCR</u>			
CMV (%)		1/8 (13)	0/4 (25)
EBV (%)		4/8 (50)	2/4 (50)
HSV (%)		1/8 (13)	2/ 4 (50)
HIV-1 (%)		0/8 (0)	0/4 (0)
Adenovirus (%)		3/8 (38)	3/4 (75)
Parvovirus (%)		1/8 (13)	1/4 (25)
Enterovirus (%)		5/8 (63)	2/4 (50)
Mean viruses per case		1.8	2.5
Overall viral prevalence		80%	

Table 12 Summary of clinical characteristics, myocardial immune cell count, and prevalence of myocardial viral genome in idiopathic dilated cardiomyopathy, stratified by the presence or absence of active myocarditis (Dallas criteria)

		Idiopathic dilated cardiomyopathy cohort	
		No active myocarditis	Active myocarditis
<u>Clinical Characteristics</u>			
Number (N)		7/7	0/7
Gender:	Male (%)	6/7 (86)	
	Female (%)	1/7 (14)	
Mean age ($\pm$ sd)		43 ($\pm$ 7.3)	
Mean LVEF ($\pm$ sd)		29 ($\pm$ 5.4)	
NYHA :	I	3/7 (43)	
	II	4/7 (57)	
	III	1/7 (14)	
	IV	0/7 (0)	
	Unknown		
Mean CD4 count copies/ml (serum) ($\pm$ sd)		N/A	
Mean CD8 count copies/ml (serum) ($\pm$ sd)		N/A	
Mean Viral load ($\pm$ sd)		N/A	
<u>Immunohistochemistry (myocardium)</u>			
Mean CD3 cell count cell count /mm ² ($\pm$ sd)		7.93($\pm$ 9.5)	
Mean CD4 cell count cell count /mm ² ($\pm$ sd)		1.7($\pm$ 4.8)	
Mean CD 8 cell count /mm ² ($\pm$ sd)		4.6($\pm$ 6.1)	
Mean CD 68 cell count /mm ² ($\pm$ sd)		5.1($\pm$ 8.3)	
<u>Prevalence of viral genome by PCR</u>			
CMV (%)		0/7 (0)	
EBV (%)		1/7 (14)	
HSV (%)		1/7 (14)	
HIV-1 (%)		0/7 (0)	
Adenovirus (%)		1/7 (14)	
Parvovirus (%)		1/7 (14)	
Enterovirus (%)		4/7 (57)	
Mean viruses per case		1.1	
Overall viral prevalence		100%	

4.4 DISCUSSION

The aim of the virology section was to establish whether there was evidence for direct viral infection of the heart in patients with HIV associated cardiomyopathy. This study is to the best of our knowledge the first study to examine the role of viral genomes in cardiac tissue in African patients with HIV-associated cardiomyopathy, heart transplant recipients, and idiopathic dilated cardiomyopathy (Sliwa 2005).

Literature has pointed to enterovirus, adenovirus, CMV and HIV-1 as the common/dominant opportunistic viral infections in the heart tissue of patients with HIV associated cardiomyopathy (Poller 2002, Wu 1992). Enterovirus, adenovirus and cytomegalovirus are also reported to be the prevalent cardiotropic viruses in the myocardium of heart transplant recipients, whilst enterovirus, adenovirus, and parvovirus are the prevailing viruses in the endomyocardial biopsy tissue of patients with idiopathic dilated cardiomyopathy (Mantke 2005, Poller 2006, Kuhl 2005).

It is well known that microscopy and immunohistochemistry have a low sensitivity for detecting the presence of viruses in the myocardium (Barbaro 2005). Infectivity has to reach hundreds of viral copies per cell before it is detectable by either one of these two methods. Numerous studies have also reported using PCR to detect viral genomic material in cardiac biopsy tissues, but it is often debatable whether the presence of genomic viral particles reflects disease-causing viral activity or a bystander effect either due to viral persistence from old infection or contamination from viraemia. In our experiments, we used a combination of PCR and immunohistochemistry to identify the viruses in the myocardium as they were considered to be the most sensitive detection techniques. Unfortunately, the immunohistochemistry assay did not yield any results in our hands, so we discuss the results of PCR in this thesis.

PCR results indicated that the myocardium of the HIV associated cardiomyopathy cohort was dominated by genomes of EBV (64%) and HSV (50%) followed by enterovirus (36%) and adenovirus (29%). EBV and HSV are common herpes viruses that are known to have latency stages; they are reactivated in immunocompromised individuals causing tissue damage. Similarly, enteroviruses are also known to have latency stages, which could have important consequences for the clinical course of the disease.

The issue of differentiation between active viral replication and latent persistence of enteroviral genomes was not addressed in this study. During enteroviral replication, the plus-strand RNA first serves as a template for the transcription of a replicate minus-strand RNA intermediate by the RNA-dependent RNA polymerase. Transcription of minus-strand RNA from the plus-strand enteroviral genomic template is the essential first step of enteroviral replication. Therefore, selective RT-PCR detection of the minus-strand RNA is an indicator of active enteroviral RNA replication which causes tissue damage (Pauschinger 1999). As we did not look for enteroviral replicative intermediates in the biopsy tissues we were unable to distinguish between current infection and latent infection in the tissues where enterovirus had been positively identified.

The low detection of HIV-1 in the tissues of the HIV-associated cardiomyopathy cases could mean (1) that HIV-1 does not infect cardiomyocytes directly in the majority of cases, resulting in an indirect mechanism for the development of HIV-1 associated cardiomyopathy (release of HIV-1 toxins leading to myocardial tissue damage or chronic infection of other viruses leading to an autoimmune reaction) or (2) that the HIV-1 copy number was too low in the serum to detect via PCR. Only minimal numbers of parvovirus (14%) and CMV (4%) were detected.

In the heart transplant recipients, the PCR results indicated that enterovirus (50%), adenovirus (50%), EBV (33%) and HSV (25%) were the

most prevalent viruses in the myocardium of these patients. The high prevalence of enterovirus, adenovirus and EBV is expected as these viruses are known to have an affinity for the myocardium (i.e., cardiotropic). The relatively low frequency of parvovirus (16.7%) and CMV (16.7%) infection was similar to the patients with HIV-associated cardiomyopathy.

In patients with idiopathic dilated cardiomyopathy, the PCR results indicated that the enterovirus (57%) and EBV (29%) were the most common viruses to infect the myocardium. Adenovirus (14%), HSV (14%), CMV (14%), and parvovirus appeared to play a minor role in these patients. In Nigeria, infection with *Toxoplasma gondii* and coxsackievirus B (an enterovirus) is thought to play a major role in the pathogenesis of idiopathic dilated cardiomyopathy in case-control studies, but this assertion has not to date been substantiated by histological studies (Falase 1979, Falase 1985). By contrast, in Kenya, where half the patients with idiopathic dilated cardiomyopathy are reported to have histological changes of a previous myocarditis, no serological evidence of a previous or recent coxsackievirus B infection or any other common viral infection has been found (Sanderson 1993). Therefore, ours is the first study to show a clear pattern of infection in patients with idiopathic dilated cardiomyopathy, with enterovirus and EBV being the common agents, and parvovirus and CMV playing a minor role.

4.5 CONCLUSION

We show for the first time that infection with Epstein-Barr virus, Herpes Simplex virus, enterovirus, and adenovirus is common in patients with HIV-associated cardiomyopathy and heart transplant recipients living in Africa. We also show that the myocardium of the idiopathic dilated cardiomyopathy cases were dominated by the Enterovirus followed by a paucity of the other viruses. We also noted that parvovirus and cytomegalovirus infect heart tissue less frequently in these patient groups. We found no obvious pattern of association with active/acute myocarditis, although the numbers in this study were small. This study has not been able to determine whether the viral genomes found represent active infection or latent infection, or indeed contamination by a viraemia at the time endomyocardial biopsy.

The absence of information on the prevalence of cardiotropic viruses in the general population makes the interpretation of these data difficult. For example, a worthy comparison may have been to take biopsies of the donor heart prior to transplantation in order to identify the most prevalent viruses in the 'normal' heart. Studies are needed to establish the prevalence of viral infection of the heart in the general population for use in these studies.

Even though PCR is the current 'gold standard' in viral detection this method also has some disadvantages. The major disadvantage of PCR is its failure to differentiate between virus in myocardium or virus in infected blood cells. When assessing endomyocardial biopsies by PCR, the potential presence of systemic virus infection or persistence must be considered. For example, enteroviruses circulate freely in the bloodstream during enteroviral-induced illnesses, hence myocardial involvement can be definitely demonstrated only if blood and myocardium were tested simultaneously and the myocardium alone was positive. Unfortunately in this study, myocardial biopsy and blood sampling occurred at varying times, thus rendering a serum-biopsy

correlation of infectivity to be unattainable. Future studies to validate our results should include the testing of serum samples taken at the same time as the endomyocardial biopsies, and the testing for replicative intermediates of enteroviruses.

University of Cape Town

CHAPTER 5

IS THE MITOCHONDRIAL
T16189C VARIANT
(RESULTING IN A 9-12 BASE
PAIR HOMOPOLYMERIC
TRACT) A RISK FACTOR
FOR HIV-ASSOCIATED
CARDIOMYOPATHY?

CHAPTER 5: IS THE MITOCHONDRIAL T16189C (RESULTING IN A 9-12 BASE PAIR HOMOPOLYMERIC C TRACT) A RISK FACTOR FOR HIV-ASSOCIATED CARDIOMYOPATHY?

5.1 INTRODUCTION

It is well established that a proportion of patients who are infected with HIV develop heart failure during the natural history of the retroviral disease. A recent study from Rwanda has shown that the prevalence of HIV associated cardiomyopathy is approximately 17.7%, with low socio-economic status, longer duration of HIV infection, advanced HIV disease, and low plasma selenium levels being the main determinants of the heart muscle disease (Twagirimukiza 2007). It is not known, however, whether genetic factors play any role in the predisposition to the development of cardiomyopathy in people who are infected with HIV.

A major advance in the study of the pathogenesis of idiopathic dilated cardiomyopathy has been the demonstration that 20-30% of cases are familial, suggesting that genetic factors may be involved in the aetiology of the disease (Ahmad 2005). In cases of sporadic dilated cardiomyopathy, genetic factors might confer increased susceptibility to disease in association with other environmental factors such as viral myocarditis. In addition to nuclear DNA abnormalities, mutations in the mitochondrial DNA may also contribute to the development of cardiomyopathy (Ozawa 1994). For example, significant amounts of mitochondrial DNA mutations have been found in cardiomyopathic myocardium (Li 1997), and when mutated mitochondrial DNA from cultured cells is introduced into mice, it results in dilated cardiomyopathy (Wallace 2000). The myocardium is dependent on mitochondria for cellular oxidative phosphorylation and is, therefore, susceptible to genetic defects that affect mitochondrial function.

The importance of subtle abnormalities in cardiac energy production is underscored by the fact that Khogali (2001) showed an

association between the T16189C variant (associated with a homopolymeric 9-12 base pair C tract) and dilated cardiomyopathy in African and British populations. Nucleotide T16189 lies in the middle of a homopolymeric-C tract where the transition from a thymine (T) to a cytosine (C) causes an uninterrupted homopolymeric C-tract of 9-12bp, which is also termed the 'OriB variant', in the displacement loop (D-loop) region of mitochondrial DNA (mtDNA) (Fig. 16).

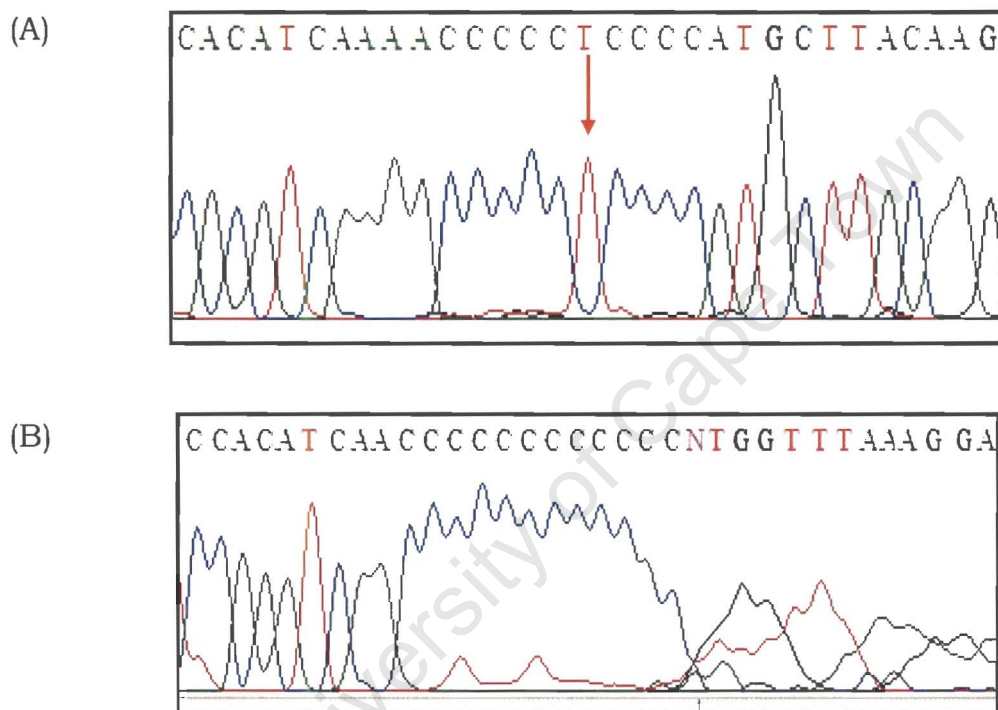


Figure 16. Electropherograms of the T16189C variant showing (A) the normal sequence (CAAAACCCCCCTCCCCATG) and (B) the variant T-C transition resulting in a homopolymeric C-tract (CAACCCCCCCCCCCCCNT) that destabilizes the DNA and causes the DNA polymerase to slip along/off the strand.

The displacement loop (D-loop) region (Figure 16) is located between the tRNA genes for proline and phenylalanine and acts as a promoter for both the heavy and the light strands of the mtDNA. It also contains essential transcription and replication elements for the whole

mtDNA genome (Wallace 1994). This non-coding region is 1124bp in length and contains hypervariable segments 1 (HSV-1) at position 16024-16383 and hypervariable segment 2 (HSV2) at position 57-372. It is considered to be a mutational hotspot due to the fact that it is the most rapidly evolving part of the mitochondrial genome; this is most probably due to the lower frequency of DNA repair, lack of protective histones as well as higher frequency of DNA replication error in mtDNA (Montazeri 2005, Wilson 1993, Croteau and Bohr 1997). Mitochondrial point mutations are associated with maternally inherited and sporadic cases of hypertrophic and dilated cardiomyopathy (Antozzi and Zeviani 1997).

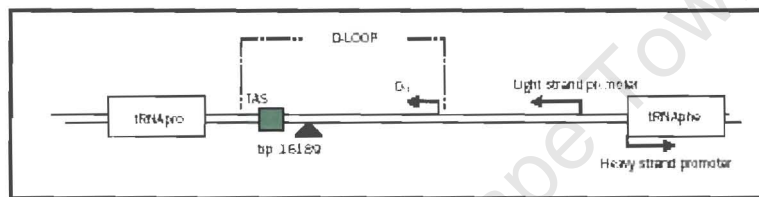


Figure 17. Schematic diagram of the D-loop region showing the T16189C variant and the origin of replication of heavy strand of mtDNA (OH) and termination associated sequence (TAS) (Khogali *et al* 2001).

As expected the association data imply that the variant increases susceptibility to idiopathic dilated cardiomyopathy, but the presence of the variant is not sufficient to cause disease. The mtDNA T16189C variant maps precisely to a novel point of origin of mtDNA replication (OriB), which makes it very likely that the variant will alter mitochondrial DNA function (Yasukawa 2005). Therefore, the critical position of the T16189C variant in the mitochondrial genome (Figure 17) suggest that the variant might have a direct role in the pathogenesis of dilated cardiomyopathy. Indeed, the T16189C variant with a 9-12 base pair homopolymeric C tract or 'OriB variant' appears to be a genetic risk factor for a other disorders, such as diabetes mellitus (Poulton 1998), and low birth weight (Casteels 1999) and is thus of medical interest.

A proportion of cases of idiopathic dilated cardiomyopathy is thought to be due to previous viral myocarditis (Sliwa 2005). Therefore, we postulated that the same variant might predispose to dilated cardiomyopathy in people with a virus-associated form of cardiomyopathy, such as HIV-associated cardiomyopathy. We tested this hypothesis in a case-control study, including patients who were involved in the endomyocardial biopsy studies reported in chapters 3 and 4.

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5.2 METHODS

5.2.1 Study design

Between 2002 and 2006 we recruited 30 cases of HIV-associated cardiomyopathy and 38 HIV-positive patients with echocardiographically normal hearts. The cases and controls were selected based on the criteria stated in Chapter 2, section 2.4.1.

5.2.2 Statistics

The HIV-associated cardiomyopathy samples were analyzed for a possible association with the T16189C genotype with a homopolymeric C-tract by using the Chi-square test; a p-value of <0.05 was considered significant. The program Statistica 7 was used.

5.3 RESULTS

The clinical characteristics of the cases and controls included in this section of the work are summarised in Table 12. Recruitment of patients meeting the entry criteria of the study (i.e., dilated cardiomyopathy in HIV positive patients that is otherwise unexplained by known causes of heart disease) proved to be challenging; we managed to recruit 30 cases of HIV associated cardiomyopathy over four years, which was 12 cases short of the target sample size for the study of 42. Thirty eight HIV positive controls with normal echocardiography examination were identified. Based on the realised number of cases and controls, the 'post hoc' statistical power of the study was calculated to be 77% at a 95% confidence level. The 30 HIV associated cardiomyopathy patients recruited were aged between 21 and 54 with a male to female ratio of 1.5:1. The controls had an age range of 20 to 52 and a male to female ratio of 1.1:1 (Table 13).

Table 13 Clinical characteristics of the HIV associated cardiomyopathy cases and HIV positive controls

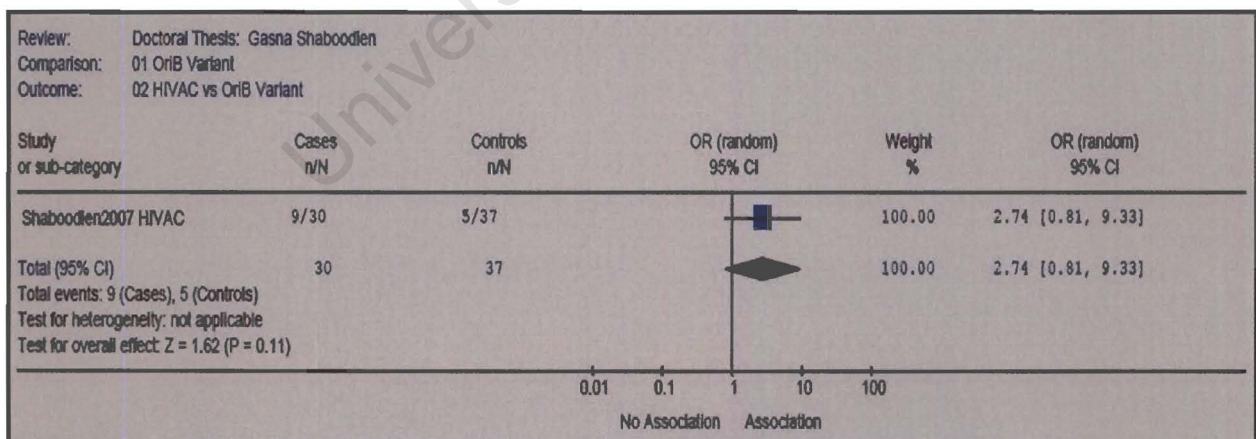
	HIVAC	HIV positive controls	P value
Median Age (IQR [‡] , ± Stdev)*	35 (27-41, ±9.3)	34 (30-38, ±7.7)	0.93
Gender			
male	18(60%)	20 (52.6%)	0.54□□
female	12 (40%)	18 (47.4%)	
Mean LVEF (± Stdev)	33.4 (±11.8)	66.7 (±11.3)	0.0001
Mean LVIDd (± Stdev)	5.9 (± 0.88)	4.4 (±0.50)	0.0001
Mean CD4 count (± Stdev)	260.7 (±213.8)	176.1 (±152.7)	0.21
RNA viral load (median)	680,774.2(92 000)	231,574.8 (66 000)	0.16
Number	30	38	

[‡]IQR: inter-quartile range; Stdev* standard deviation; □□Mann-Whitney non-parametric test
LVIDd- left ventricular internal dimension in diastole

We tested the hypothesis that the OriB variant may predispose to dilated cardiomyopathy in HIV positive individuals based on the putative genetic association of this variant with idiopathic dilated cardiomyopathy, a proportion of which may be triggered by viral myocarditis.

We found the OriB variant occurred at a frequency of 30% (9/30) in the HIV-associated cardiomyopathy cases and in 13.5% of the HIV positive controls (5/37) with no cardiomyopathy. We also found that of the four length variants found (i.e., C9, C10, C11, and C12), heteroplasmic length C10 occurred most commonly in the HIV-associated cardiomyopathy patients at a frequency of 20% (6/30); the frequency of the C10 heteroplasmic length variant in the HIV positive cases without dilated cardiomyopathy occurred at a frequency of 8% (3/37).

To test the degree of association between the observed results and the expected results (based on the hypothesis being tested) we used the chi-square statistical test. The statistical tests performed indicated that the OriB variant was not significantly associated with HIV-associated cardiomyopathy (Odds ratio 2.74, 95% Confidence interval 0.81 – 9.33, $P=0.11$) (Fig. 18).



Horizontal lines, 95% credible interval

Figure 18 Forest plot indicating the frequency of the OriB variant in cases and controls

5.3.1 Other polymorphisms

Numerous other variants were also found in the 10bp region and are presented in Table 14. No base changes were observed at mitochondrial positions 16184, 16186, 16190 and 16192. After the T16189C variant, the C16187 variant occurred most frequently in both cohorts at 43.3% (13/30) in the HIV-associated cardiomyopathy cohort and at 51.3% (19/37) in the HIV positive cohort. There were no statistical significant differences between cases and control for these additional polymorphisms.

Table 14 Frequency of other polymorphisms in the 10bp D-loop region (16184bp-16193bp) in cases and controls

	16184bp-16193bp	HIVAC N, (%)	HIV+ N, (%)	χ^2	<u>p-value</u>	<u>OR</u>
Wild Type	CCCCCTCCCC					
C16185T	CTCCCTCCCC	2 (6.66)	1 (2.7)	0.03	<u>0.58</u>	<u>2.57</u>
C16187T	CCCTCTCCCC	13 (43.3)	19 (51.3)	0.42	<u>0.52</u>	<u>0.72 (0.25-2.13)</u>
C16188G	CCCGCTCCCC	4 (13.3)	4 (10.8)	0	<u>1.0</u>	<u>1.27 (0.21-7.49)</u>
C16188A	CCCCATCCCC	1 (3.3)	0	0.01	<u>0.45</u>	<u>Undefined</u>
T16189C	-----C-----	21(70)	23 (62.2)	0.44	<u>0.50</u>	<u>1.42 (0.45-4.49)</u>
C16191G	CCCCCTCGCC	1 (3.33)	0	0.01	<u>0.45</u>	<u>Undefined</u>
C16192T	CCCCCTCCTC	0	1 (2.7)	0.0	<u>1.0</u>	<u>0 (0-48)</u>
C16193G	CCCCCTCCCG	1 (3.33)	1 (2.7)	0.33	<u>1.0</u>	<u>1.24</u>

K-constant, χ^2 – chi square; p- value; OR- odds ratio

5.4 DISCUSSION

To the best of our knowledge, this is the first study of genetic susceptibility factors in HIV-associated cardiomyopathy. This study shows a trend towards an increased frequency of the OriB variant in patients with HIV-associated cardiomyopathy (30%) compared to HIV positive patients without cardiomyopathy (13.5%), but this trend is not statistically significant (OR 2.74, CI 0.7-11.78). It is uncertain whether the lack of significant difference between cases and controls are related to inadequate statistical power (i.e., false negative) or a real absence of an association between the OriB variant and HIV-associated cardiomyopathy.

There are differences between this study and the original study that found an association between the OriB variant and dilated cardiomyopathy (Khogali 2001). Khogali (2001) studied patients with idiopathic dilated cardiomyopathy, whereas we studied cases of HIV-associated cardiomyopathy. It is likely that some cases of idiopathic dilated cardiomyopathy result from unrecognized viral myocarditis early in life, a factor that resulted in our postulate that the same variant may predispose to an inflammatory cardiomyopathy such as HIV-associated cardiomyopathy. However, idiopathic dilated cardiomyopathy is likely to represent a more heterogeneous group than patients with HIV-associated cardiomyopathy. Furthermore, we have shown in a previous chapter that HIV-associated cardiomyopathy is characterized by greater frequency of acute myocarditis than idiopathic cardiomyopathy, which underlies the fact that these are likely to be different disease entities.

In the Khogali study, the finding of association of the OriB variant with idiopathic dilated cardiomyopathy in black Africans was based on 22 cases and 19 controls, a sample size that is even smaller than the present study. It is well recognised that smaller studies may be associated with false positive findings (Mayosi 2005). The putative genetic association of the ACE I/D polymorphism with myocardial

infarction is a salutary case in this regard. A series of smaller studies showed extreme, albeit conflicting findings of association between ACE I/D genotype and myocardial infarction (Keavney 2000). A larger, and adequately powered validation study was required to produce a more realistic estimate of the effect of the ACE I/D polymorphism on the risk of myocardial infarction. Indeed a study involving thousands of cases and thousands of control showed no significant association between the ACE I/D polymorphism and the risk of myocardial infarction (Keavney 2000).

Our finding therefore, raises concern about the validity of the putative association between the OriB variant and cardiomyopathy in the original small study by Khogali (Khogali 2001). One way of resolving this uncertainty was to conduct a validation study in an adequately powered study of cases and controls with cardiomyopathy, and to pool the findings with all relevant studies in a meta-analysis. The replication experiment and the meta-analysis of the genetic association of the OriB variant and cardiomyopathy are presented in Chapter 6.

CHAPTER 6

A SYSTEMATIC REVIEW
AND META-ANALYSIS OF
THE ASSOCIATION OF THE
MITOCHONDRIAL T16189C
VARIANT (RESULTING IN A
9-12 BASE PAIR
HOMOPOLYMERIC TRACT)
WITH CARDIOMYOPATHY

CHAPTER 6: A SYSTEMATIC REVIEW AND META-ANALYSIS OF THE ASSOCIATION OF THE MITOCHONDRIAL DNA T16189C VARIANT (WITH A 9-12 BASE PAIR POLY C TRACT) WITH CARDIOMYOPATHY

6.1 INTRODUCTION

We hypothesised that the OriB variant, originating from the mitochondrial DNA T16189C polymorphism, which is putatively associated with an increase risk of the development of cardiomyopathy in the general population, may predispose to the development of cardiomyopathy in people living with HIV. The putative association of the OriB variant with dilated cardiomyopathy in black Africans is based on a single report involving 22 cases and 19 controls (Khogali 2001). Our study of 30 cases of HIV-associated cardiomyopathy and 37 HIV infected patients without cardiomyopathy failed to show a significant association of the mtDNA T16189C variant with cardiomyopathy (Chapter 5). We have conducted a replication of the Khogali (2001) study in a larger sample of cases and controls; all the results of other studies that have examined association of the OriB variant with cardiomyopathy were pooled in a meta-analysis.

We employed the methods of the Human Genome Epidemiology Network (HuGE Reviews Handbook version 1.0, URL 3) in this review to summarise all studies reporting on the association of the T16189C variant with dilated cardiomyopathy. HuGE reviews are intended to be systematic, peer-reviewed synopses of epidemiologic aspects of human genes and are prepared following a standard internationally recognized procedure of The HuGENet™. These reviews were introduced as a means of integrating evidence from human genome epidemiologic studies of the impact of human genetic variation on health and disease. They describe the systematic literature reviews and meta-analysis as a mechanism for evaluating the overall effect of a polymorphism and/or genes.

HuGE reviews differ from traditional literature reviews in that they are in themselves research projects, with a pre-specified protocol which consists of an exhaustive literature search strategy, criteria of studies included in the review and the application of methodological rigor to the review process. The use of explicit, systematic methods in reviews limits bias (systematic errors) and reduces chance effects, thus providing more reliable results upon which to draw conclusions and make decisions.

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6.2 METHODS

6.2.1 Replication study

6.2.1.1 Case control study

Between 2002 and 2006, 99 cases with idiopathic dilated cardiomyopathy and 117 normal population controls representing the South African background population were identified. Cases and control were selected as per Chapter 2, section 2.4.2.

6.2.2 Systematic review and meta-analysis

6.2.2.1 Review inclusion and exclusion criteria

Case-control and cohort studies investigating the association of the OriB variant with idiopathic dilated cardiomyopathy were eligible for inclusion. Studies without an appropriate control group were excluded.

6.2.2.2 Study identification and eligibility

Two independent observers (Gasnat Shaboodien [G.S.], and Mark Engel [M.E.]) identified studies from Pubmed and ISI Web of Knowledge(URL4) (each from its earliest date until September 2007) using a predetermined search strategy incorporating the following terms: ((dilated cardiomyopath*) AND (polymorph* or mutation* or variant* or genotype* or gene* or allele*) AND mitochondri*). We also searched the MITOMAP database (URL5) using the term "T16189C". This process was complemented by reviewing citations, and searching in Google Scholar (URL6). We placed no restriction on the language of publication. Titles and abstracts were screened, and full text articles obtained from potentially eligible reports. We excluded animal studies, reviews, case reports, editorials and meeting abstracts.

6.2.2.3 Data extraction

We (G.S., M.E.) independently recorded the following information from each study: year of publication, study design, origin and demographics of cases and controls, matching procedures, diagnostic criteria, and information on variants identified. For studies including subjects from differing ethnic backgrounds/geographical locations, data were extracted separately where possible. We also evaluated blinding of relevant personnel. A third reviewer, (Bongani M Mayosi [B.M.M]) served as arbitrator where necessary.

6.2.2.4 Data synthesis

Odds Ratios (ORs) for the OriB variant as a risk factor for dilated cardiomyopathy from individual studies were combined by random-effects meta-analysis according to the Mantel-Haenszel method. For our study we chose the random-effects model as the method of choice as this model is more appropriate when heterogeneity is present. Random effects incorporate an estimate of the between-study variance and tend to provide wider confidence intervals, when the results of the constituent studies differ among themselves (Ntais 2004). Heterogeneity was evaluated using the χ^2 -based Q statistic and was considered significant for $p < 0.10$.

6.3 RESULTS

6.3.1 Replication study

The demographic details of the cases and controls are summarized in Table 15. All patients were aged between 18 and 65 (mean age 52) with a male to female ratio of 2.2:1; patients with secondary causes of cardiomyopathy (such as hypertension, valvular heart disease, and coronary artery disease) were excluded from the study. Convenient sampling was done but, where possible, an attempt was made to match the cases and controls for age and gender.

In order to have a study with 80% power at a 95% confidence interval the statistics indicated that we needed to recruit 43 cases and 43 controls; this study would thus have sufficient power to detect an association if one existed. Over the four year period we recruited 99 idiopathic dilated cardiomyopathy cases and 117 controls; thus more than double the number of cases and controls that were needed had been recruited. The post-hoc power calculation was determined to be 99.7% at an α of 5%. We were confident that, should an association exist, the study was sufficiently powered to detect this association.

Table 15 Summary of demographic details of cases and controls

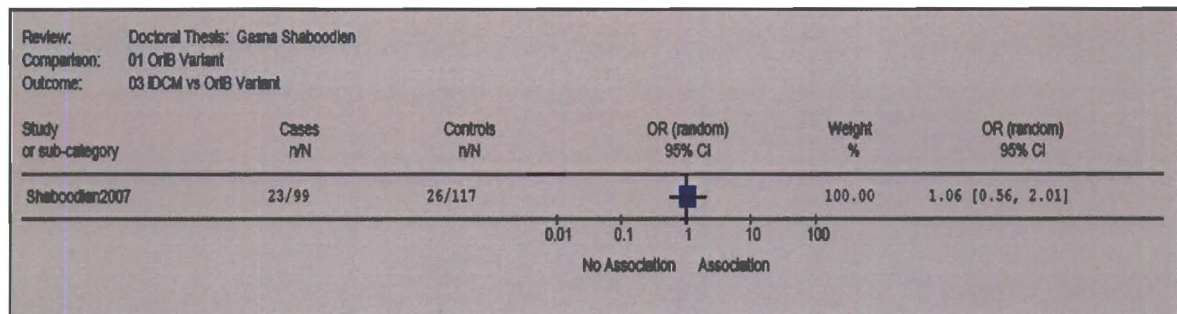
	iDCM	Background	P value
Mean Age ($\pm$ SD)	59 ($\pm$ 16.4)	47($\pm$ 6.7)	<0.0001 [□]
Mean LVEF ($\pm$ SD)	33.97 ($\pm$ 11.0)	n/a	
Ethnicity	Black	Black	
Population source	Hospital	Genetics laboratory	
Sampling	Incident cases	Convenient	

Stdev-standard deviation

[□]Mann-Whitney non-parametric test

We found the OriB variant presented at a frequency of 23% in idiopathic dilated cardiomyopathy cohort and 22% in the background

population, thus no statistical significance was found between the two groups (OR 1.06, CI 0.56-2.01) (Figure 19).



Horizontal lines, 95% confidence interval; a square block represents a study; the area of the block indicates the weight assigned to that study; the horizontal line depicts the confidence interval; the size of the block indicates the studies with larger weight

Figure 19 Forest plot indicating the frequency of the OriB variant in cases and controls

We also found that different length variants were generated as a result if the T16189C transition; viz heteroplasmic length C10, C11, C12 and C13 (Figure 16). Heteroplasmic length C12 occurred more commonly in both cohorts at a frequency of 12.1% (12/99) in the idiopathic dilated cardiomyopathy cohort compared with 7.7% (9/117) in the South African background population; the frequencies were not significantly different between the two groups (OR 1.66, CI 0.62-4.5).

A summary of all variations found the 10base pair region are presented in Table 16.

Table 16 Summary of sequencing variations and statistical data found in the 10bp D-loop region (16184bp-16193bp) in the idiopathic dilated cardiomyopathy patients and background population.

		HIV-DCM+(%)	HIV-DCM- (%)	χ^2	p-value	OR 95% CI
C16184		K	K	N/A	N/A	N/A
C16185T	C T C C C T C C C C	3 (3.0)	4 (3.4)	0.05	1.0	0.88 (0.13-5.36)
C16186		K	K	N/A	N/A	N/A
C16186G	C C G C C T C C C C	1 (1.0)	K	0.01	0.46	Undefined
C16187T	C C C T C T C C C C	53 (53.5)	55 (47)	0.91	0.34	1.31 (0.73-2.30)
C16188G	C C C G C T C C C C	18 (18.2)	20 (17.1)	0.04	0.83	1.08 (0.50-2.3)
T16189A	C C C C C A C C C C	1 (1.0)	K	0.01	0.46	Undefined
T16189C	C.....	78 (78.8)	83 (70.9)	0.16	0.69	0.88 (0.48-1.64)
C16190		K	K	N/A	N/A	N/A
C16191		K	K	N/A	N/A	N/A
C16192T	C C C C C T C C T C	3 (3.0)	3 (2.6)	0.04	1.0	1.19 (0.16-9.07)
C16193G		K	K	N/A	N/A	N/A
cohort total		99	117			

K-constant; N/A-not applicable

6.3.2 Systematic review and meta-analysis

The process by which articles were selected for our systematic review is detailed in the Appendix 3. We searched three databases which yielded 151 citations. Duplicate records across the three databases were deleted, leaving 47 articles deemed to be potentially relevant. Titles and abstracts were evaluated by two observers working independently and 41 studies were excluded; following full-text scrutiny, a further four publications were excluded. Reasons for exclusion were outcomes other than DCM (n=5), other polymorphisms (n=24), not gene-association study (n=3) and reviews/editorials (n=13).

6.3.2.1 Characteristics of the included studies

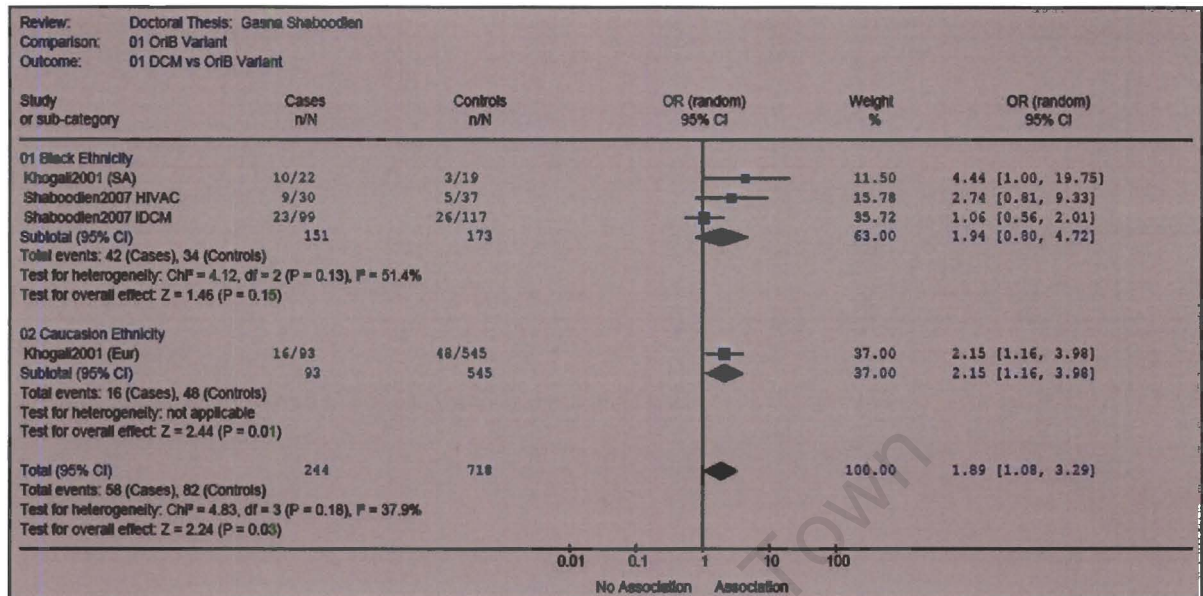
Only one publication (Khogali 2001) satisfied our inclusion criteria. Together with our unpublished data, the eligible studies comprised three distinct study populations contributing a total of 962 subjects for

analysis with a median sample size of 162. All studies were case-controlled, conducted in either European Caucasian or African ethnic groupings. All included studies characterised the cases according to standard criteria (WHO classification, URL7). Controls varied in composition and, where stated, ranged from healthy members of the general population to hospital inpatients. None of the studies reported on baseline characteristic differences between comparison groups. Blinding of genotyping was not mentioned or absent. Additional information was received through direct contact with two of the study authors (B M Mayosi and J Poulton) of the Khogali (2001) paper.

6.3.2.2 Meta-analysis

The combined meta-analysis revealed a statistically significant association of the OriB variant with dilated cardiomyopathy, (MH-OR 1.89; 95% CI 1.08; 3.29; P=0.03). Sub-group analysis according to ethnicity, however, showed that the OriB variant was not found to be significantly associated with dilated cardiomyopathy in participants of black African ancestry (MH-OR 1.94, 95% CI 0.8; 4.72, P=0.15). The study with the Caucasian population showed a significant association between the variant and dilated cardiomyopathy (MH-OR 2.15, 95% CI 1.16; 3.98, P=0.01).

No statistically significant heterogeneity was found across the studies ($p > 0.1$). The I^2 statistic was consistent with these results (Figure 20).



Horizontal lines, 95% confidence interval; a square block represents a study; the area of the block indicates the weight assigned to that study; the horizontal line depicts the confidence interval; the size of the block indicates the studies with larger weight; the diamond shape depicts the combined effect of the studies

Figure 20 Forest plot displaying the relationship between the OriB variant and dilated cardiomyopathy risk in a systematic review and meta-analysis.

6.4 DISCUSSION

There is a paucity of studies that have examined the putative genetic association of the mitochondrial T16189C variant with a homopolymeric C tract (i.e., OriB variant) with dilated cardiomyopathy. Our results show that in the African population, smaller studies show a promising association (i.e., Khogali 2001 and Shaboodien 2007 in chapter 5), while a large replication study was neutral in this group. The only information that is available among Caucasians is based on the original study of Khogali *et al* which showed an association in a British cohort. Subsequent studies in Caucasians, such as that of Ruppert *et al* (2004), unfortunately did not examine the OriB variant, but rather considered the T16189C variant without reference to the poly C tract. Therefore, the initial finding of an association among Caucasians remains to be replicated in an appropriately designed and powered study.

The analysis of small subgroups in the original report of Khogali (i.e., in the African subjects with 22 cases and 19 controls), shows an extreme association with an odds ratio of 4.44, whereas the larger replication study shows a neutral effect with an odds ratio of 1. This trend, in which smaller genetic association studies show extreme effects which are not observed in larger studies, has been noted, for example, in the case of the putative association of the ACE I/D genotype with myocardial infarction (Keavney 2000). The published smaller studies, and in particular retrospectively defined sub-group analyses, showed extremely promising results for association of genotype with myocardial infarction. The large scale study of this hypothesis that involved 5000 cases and 6000 controls showed no significant association of ACE genotype with myocardial infarction (Keavney 2000). A replication study of the putative association of the mitochondrial T161869C variant with cardiomyopathy in Caucasians is required. Such a study must examine the OriB variant, in which the polyC tract is uninterrupted, rather than

just the T16189C variant which may be interrupted by other polymorphisms in the region.

It is also possible that the association of the OriB variant with dilated cardiomyopathy may be dependent on ethnic background, with the variant being more relevant among Caucasians than black Africans. There are other examples of the ethnic variation in genetic susceptibility factors for multifactorial disease. For example, nucleotide-binding oligomerization domain protein 2 (NOD 2) is a major susceptibility gene for Crohn disease among Jewish people (Lesage 2002, Sugimura 2003), but not among the Japanese (Yamazaki 2002). This question of ethnic specificity of the mitochondrial T16189C variant with a poly-C tract in cardiomyopathy remains to be resolved by means of an appropriately designed replication study in the Caucasian population.

The results of our experimental study and the meta-analysis demonstrate that studies have to be carefully designed, adequately powered and interacting population specific factors have to be taken into account in order to produce reliable results on putative association of genetic factors with multifactorial traits (Mayosi 2005). This study shows that the mitochondrial T16189C variant on a homopolymeric C tract is not a risk factor for HIV-associated and idiopathic dilated cardiomyopathy in black Africans.

CHAPTER 7

CONCLUSION

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7.1 AIMS AND OBJECTIVES

This study was designed to elucidate the pathogenesis of HIV-associated cardiomyopathy by means of histological, virological, and genetic methods. The central hypothesis was the HIV-associated cardiomyopathy was likely to be a form of myocarditis that affected a sub-set of people who were genetically predisposed to develop heart muscle disease when infected with cardiotropic viruses. The objectives were to (1) define the histological abnormality in HIV-associated cardiomyopathy using light microscopy, immunohistochemistry, and electron microscopy, (2) to establish whether cardiotropic viruses played a role in the pathogenesis of HIV associated cardiomyopathy, and (3) to determine if mitochondrial variant T16189C with a poly-C tract (i.e., the OriB variant) predisposed to cardiomyopathy in people who are infected with HIV.

7.2 STUDY DESIGN

We used a series of case control/comparison experiments to meet the objectives of the study. To address the first two objectives, we conducted an endomyocardial biopsy study in which we compared the histological and virological changes in HIV-associated cardiomyopathy with patients with idiopathic dilated cardiomyopathy and heart transplant recipients (the 'controls'). The third objective was addressed by comparing the prevalence of the mitochondrial OriB variant in HIV-associated cardiomyopathy, compared to people with HIV infection and echocardiographically normal cardiac structure and function. The latter study was confined to cases and controls of black African ancestry in order to avoid confounding of the results of the genetic association by population stratification.

7.3 FINDINGS

7.3.1. Histology

This study contributes to new knowledge in at least four ways. First, we show that active/acute myocarditis accounts for a minority of patients with HIV-associated cardiomyopathy (approximately 25%) in Africans. This is in contrast to others who have suggested that active/acute myocarditis is the pathological hallmark of HIV-associated cardiomyopathy. Second, there is a marked increase in CD8+ lymphocytes in the heart tissue of patients with HIV-associated cardiomyopathy. This response may be indicative of either direct or indirect viral infection of the cardiac muscle; a high prevalence of viral infection is confirmed in a subsequent chapter, but the preponderance in CD8+ T cells is not seen in other heart transplant recipients and idiopathic dilated cardiomyopathy who also have evidence of viral infection in heart tissue. Therefore, this pattern of cellular abnormality appears to be specific to patients with HIV-associated cardiomyopathy. Third, we confirm that HIV-associated cardiomyopathy is associated with an extremely poor prognosis in patients who are not treated with anti-retroviral drugs. Finally, based on the nuclear changes found in the biopsy tissues of all three groups with varying degrees of myocardial inflammation, we postulate that inflammatory cardiomyopathy may be characterised by an acquired form of a nuclear laminopathy.

7.3.2. Virology

We show for the first time that infection with Epstein-Barr virus, Cytomegalovirus, herpes simplex virus, enterovirus, and adenovirus is common in patients with HIV-associated cardiomyopathy, idiopathic dilated cardiomyopathy and heart transplant recipients living in Africa. We also note a consistent pattern across these groups that parvovirus and CMV

infect heart tissue less frequently than the former viruses. We found no obvious pattern of association of viral infection with active/acute myocarditis, although the numbers in this study were small. This study has not been able to determine whether the viral genomes found represent active infection or latent infection, or indeed contamination by a viraemia at the time endomyocardial biopsy.

7.3.3. Mitochondrial Genetics

We found that the mitochondrial T16189C variant with a poly-C tract (i.e., OriB variant) was not associated with an increased risk of HIV-associated cardiomyopathy. Furthermore, a larger replication study involving 99 cases and 117 controls failed to confirm the association of the OriB variant with idiopathic dilated cardiomyopathy in Africans. A meta-analysis of all studies published to date on the association, however, showed that, overall the OriB variant increased the risk of development of cardiomyopathy by 9% (OR 1.09; 95%CI 1.08,3.29;P=0.03). The association was only present in patients of Caucasian background. Therefore, the OriB variant may be an example of an ethnic-specific genetic risk factor for cardiomyopathy.

7.4 FUTURE WORK

This study lays the basis for larger studies of the pathogenesis of HIV-associated cardiomyopathy, and indeed dilated cardiomyopathy in general. The relatively small sample size of the endomyocardial biopsy study indicates that the histology and virology studies need to be conducted on larger sets of patients to corroborate the findings in this study. There is also a need to compare these findings with normal cardiac tissue from people without structural heart disease.

The finding of nuclear abnormalities in the three patient groups suggests that this may be a marker of inflammatory heart muscle disease. There is a need for further characterisation of the nuclear

lamina changes, and the determination of their pathogenic role in cardiomyopathy. These studies will require electron microscopic and immunohistochemistry studies in diverse groups of patients with acquired heart muscle disease, including normal controls.

This study has provided preliminary information on the potential role of viral infection in the pathogenesis of HIV-associated and idiopathic dilated cardiomyopathy. We have provided evidence that the myocardium of patients with HIV-associated cardiomyopathy, idiopathic dilated cardiomyopathy, and heart transplant recipients is infected mainly with Epstein-Barr virus, enterovirus, adenovirus, and herpes simplex virus. The pathogenetic role, however, of viral infection is not clear from our study. These studies need to be extended to investigations of the replicative state of the viral infection, and simultaneous serum studies to exclude the potential spurious role of contamination by viraemia are required. Larger studies are required to determine whether acute/active myocarditis is associated with a specific viral pattern, and to examine its impact on outcome.

The genetic basis of susceptibility to HIV-associated cardiomyopathy needs to be investigated through epidemiologic and molecular methods. Studies of twins, siblings, and families with HIV may provide epidemiological evidence of familial clustering as an measure of the size of genetic effect, if any, in this condition. Ideally, such genetic epidemiologic evidence of inherited susceptibility to the development of disease is required before embarking upon expensive molecular genetic investigation. The latter studies require adequately powered studies of well characterised patients with the disorder of interest. There is therefore a need to establish large multicentre cohort studies of patients with HIV-associated cardiomyopathy in order to address the questions related to pathogenesis of this disorder.

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APPENDICES

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APPENDICES

Appendix 1 Fixatives

Light Microscopy (LM)

Formalin

	1L	2L
NaCl	9g/L	18g/L
tapH ₂ O	900ml	1800ml
Formalin (40%)	100ml	200ml
	-----	-----
	1000ml	2000ml

Transmission electron microscopy (TEM)

2.5% Gluteraldehyde

(1) Phosphate buffer

Solution A NaH₂PO₄
27.68g/litre

Solution B Na₂HPO₄
28.38g/litre

Solution C

Combine 140ml of A and 360ml of B = 500ml (ph 7.2)

(2) Add 100 ml of stock Gluteraldehyde (25%) to solution C and make up to 1L with phosphate buffer . ∴ 2.5% gluteraldehyde

Immunohistochemistry (IHC)

2% H₂O₂

100ml dH₂O and 2ml H₂O₂

Thus Goat serum 1:20 (require 200ul)
(10ul serum + 180 dH₂O)

Prepare antibodies (Ab)(*primary antibody*)

Thus CD68 (1:100)

Thus CD8 (1:50)

Thus CD4 (1:50)

Thus p24 (1:10) *was reconstituted in 1000ul Ab diluent/dH₂O*

Chromogen (Dab substrate)

2 drops Dab 2ml solution

Fluorescent Microscopy

Sudan Black

12 mg crystals in 4ml=0.3%

Then filter

Secondary antibody

(Donkey x goat- A** 488)

1:100 dilution (200ul per slide)

8ul secondary antibody in 792ul PBS= 800ul

Flash freezing

1L liquid nitrogen from the storage tank

Making liquid nitrogen

Place acetone at -20°C. Combine acetone and dry ice "chips" to make the snapfrozen reagent mix. Mixture will be ice cold for 15 minutes and will evaporate.

Appendix 2 Raw data

Table 1 Summary of viral presence in the endomyocardial biopsies of HIVAC patients as detected by PCR

HIVAC	ENTERO	CMV	EBV	HSV	PARVO	ADENO	HIV-1
HIVAC 1	-	-	+				-
HIVAC 2	+	-	-	+	-	-	-
HIVAC 3	-	-	+	-	-	-	+
HIVAC 4	-	-	+	+	-	-	-
HIVAC 5	-	-	-	+	-	-	-
HIVAC 6	+	-	+	+	-	-	+
HIVAC 7	-	-	-	+	-	-	-
HIVAC 8	-	-	+	+		-	+
HIVAC 9	-	+	+	+	-	-	+
HIVAC 10	+	-	-	-	+	+	-
HIVAC 11	+	-	+	-	+	+	-
HIVAC 12	-	-	+	-	-	-	-
HIVAC 13	-	-	-	-	-	+	-
HIVAC 15	+	-	+	-	-	+	-

Key

CMV-cytomegalo-, **EBV**- Epstein Barr-, **HSV**- Herpes Simplex-, **Parvovirus**

EMB- endomyocardial biopsy, **n/a** – not available, (+) positive (-) negative

Table 2 Summary of viral presence in the endomyocardial biopsies of heart transplant recipient patients as detected by PCR

TRANSPLANTS	ENTERO	CMV	EBV	HSV	PARVO	ADENO	HIV-1
HIVCONTROL 1			+	-	-	-	-
HIVCONTROL 2		-	-	-	-	-	-
HIVCONTROL 3		-	+	-	+	+	-
HIVCONTROL 4		-	-	-	-	+	-
HIVCONTROL 5	+	-	+	-	+	+	-
HIVCONTROL 6	+	-	-	+	-	-	-
HIVCONTROL 7	+		+	-	+	-	-
HIVCONTROL 8	+	-	-	-	-	+	-
HIVCONTROL 9	+	-	-	+	-	-	-
HIVCONTROL 12	+	-	+	+	-	+	-
HIVCONTROL 13	+	+	+	-	-	+	-
HIVCONTROL 22	-	-	-	-	-	-	-

Key

CMV-cytomegalo-, **EBV**- Epstein Barr-, **HSV**- Herpes Simplex-, **Parvovirus**
EMB- endomyocardial biopsy, n/a not available, (+) positive (-) negative

Table 3 Summary of viral presence in the endomyocardial biopsies of idiopathic dilated cardiomyopathy patients as detected by PCR

IDCM	ENTERO	CMV	EBV	HSV	PARVO	ADENO	HIV-1
HIVCONTROL 10			+			-	-
HIVCONTROL 11	-	-		+	-		-
HIVCONTROL 15	+	-	-	-	-	-	-
HIVCONTROL 16	-	-	-	-	-	+	-
HIVCONTROL 19	+	-	-	-	-	-	-
HIVCONTROL 21	+	-	-	-	-	-	-
HIVCONTROL 25	+	-	-	-	+	-	-

Key

CMV-cytomegalo-, **EBV**- Epstein Barr-, **HSV**- Herpes Simplex-, **Parvovirus**

EMB- endomyocardial biopsy, **n/a**- not available

(+) positive

(-) negative

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Table 4 Summary of clinical characteristics of myocarditis positive HIV associated cardiomyopathy cases

	HIVAC										
	Case1	Case3	Case4	Case5	Case6	Case7	Case8	Case11	Case13	Case14	Case15
Age	44	40	42	39	53	32	34	26	47	63	41
Gender	female	Male	male	female	male	female	male	female	male	male	male
LVEF	?	28	21	31	45	32	25	38	26	38	25
CD3 (mm ³)	14	1	49	23	10	9	0	14		23	52
CD8(mm ³)	15	1	278	5	10	0	0	15		39	-
EMB date	10/10/ 2003	04/02/20 04	26/01/2004	05/02/2004	16/02/20 04	12/08/2004	16/08/2004	14/04/2005	02/06/20 05	09/06/2005	24/06/2 005
Vital statistics	-	01/06/20 06	01/05/2005	01/09/2004	01/02/20 05	Alive	03/03/2005	07/2006	04/2006	01/2006	-
Duration of illness post EMB	-	28 months	15 months	18 months	22 months	34 months	7 months	27 months	10 months	7 months	
Viral genomes**											
CMV	-	-	-	-	-	-	-	-	-	NO DNA	NO DNA
EBV	+	+	+	-	+	-	+	+	-		
HSV	-	-	+	+	+	+	+	-	-		
HIV-1	-	+	-	-	+	-	+	-	-		
PARVO	-	-	-	-	-	-	-	+	-		
ADENO	-	-	-	-	-	-	-	+	+		
ENTERO	-	-	-	-	+	-	-	+	-		

** viral genomes in the endomyocardial biopsy

Table 5 Summary of clinical characteristics of myocarditis negative HIV associated cardiomyopathy cases

		HIVAC		
		Case2	Case9	Case10
Age		27	38	54
Gender		female	male	male
LVEF		?	31	22
EMB date		01/11/2003	19/08/2004	31/03/2005
	CD3 (mm ³)	8	10	0
	CD8 (mm ³)	11	13	0
Vital statistics		Alive	Alive	01/03/2005
Duration of illness post EMB		43 months	34 months	9 months
Viral genomes				
	CMV	-	+	-
	EBV	-	+	-
	HSV	+	+	-
	HIV-1-1	-	+	-
	PARVO	-	-	+
	ADENO	-	-	+
	ENTERO	+	-	+

Table 6 Summary of clinical characteristics of myocarditis positive heart transplant recipient cases

	Cont1	Cont2	Cont3	Cont4	Cont5	Heart Transplant recipients		Cont8	Cont9	Cont12	Cont13	Cont22
						Cont6	Cont7					
Age	45	26	42	44	26	52	50	44	69	63	51	56
Gender	Fmale	female	male	male	female	male	male	Male	male	male	male	male
LVEF	52	59	51	49	57	49	59	40	49	39	59	53
CD3 (mm ³)	-	0	-	13	12	-	8	-	5	20	1	44
CD8 (mm ³)	-	0	-	3	0	-	6	-	4	22	0	11
EMB date	31/05/2004	31/05/2004	09/06/2004	09/06/2004	14/06/2004	22/11/2004	30/08/2004	22/11/2004	22/11/2004	24/01/2005	24/01/2005	17/03/2005
Vital status	Alive	Died	Alive	Alive	alive	alive	Alive	Alive	Alive	Alive	Alive	Alive
Duration of illness post EMB	3 months	3 months	24 months	24 months	2 months	31 months	34 months	31 months	31 months	29 months	29 months	27 months
Viral genomes												
CMV	+	-	-	-	-	-	-	-	-	-	+	-
EBV	-	-	+	-	+	-	+	-	-	+	+	-
HSV	-	-	-	-	-	+	-	-	+	+	-	-
HIV-1	-	-	-	-	-	-	-	-	-	-	-	-
PARVO	-	-	+	-	+	-	+	-	-	-	-	-
ADENO	-	-	+	+	+	-	-	+	-	+	+	-
ENTERO	-	-	-	-	+	+	+	+	+	+	+	-

Table 7 Summary of clinical characteristics of idiopathic dilated cardiomyopathy cases with myocarditis

	Cont10	Cont11	Cont15	Cont16	Cont19	Cont21	Cont25
Age	45	54	48	48	46	42	39
Gender	female	male	male	male	Male	female	male
LVEF	27	30	27	27	30	21	27
Myocarditis-Histology (Dallas criteria)	Borderline	Borderline	Borderline	Borderline	Borderline	Borderline	Borderline
Myocarditis-immunohistochemistry	Negative	Negative	Negative	Negative	Positive	Negative	Negative
CD3 (mm ³)	1	5	0	0	20	7	1
CD8 (mm ³)	0	16	0	0	9	0	1
EMB date	20/01/2005	13/01/2005	24/02/2005	24/02/2005	01/03/2005	10/03/2005	09/06/2005
Vital statistics	Unknown	alive	unknown	unknown	unknown	alive	alive
Duration of illness post EMB	-	29 months	28 months	28 months	-	27 months	24 months
Viral genomes							
CMV	-	-	-	-	-	-	-
EBV	+	-	-	-	-	-	-
HSV	-	+	-	-	-	-	-
HIV-1-1	-	-	-	-	-	-	-
PARVO	-	-	-	-	-	-	+
ADENO	-	-	-	+	-	-	-
ENTERO	-	-	+	-	+	+	+

Appendix 3

