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Whole Blood Mitochondrial DNA Depletion in Human Immunodeficiency Virus-Infected Children

by

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Student number: VWTGEO001

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

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7th May 2010

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DECLARATION

I, George Frederick van der Watt, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Lastly I would like to thank all of those beautiful children who gave to make this work possible. A number of them have passed on and others continue to survive, many in very difficult circumstances. It is my sincerest hope that science in this field will continue to advance and that they can one day be saved from this dreadful disease that none of them deserve.

George van der Watt

May 2010

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Abstract for MMed Chemical pathology Dissertation

George F van der Watt

Division of Chemical Pathology, Red Cross Childrens' Hospital, National Health Laboratory Service and University of Cape Town

Whole Blood Mitochondrial DNA Depletion in Human Immunodeficiency-virus Infected Children

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Background: Nucleoside reverse transcriptase inhibitors (NRTIs) interfere with mitochondrial DNA polymerase gamma causing significant toxic effects, including fatal lactic acidosis. Little is known about mitochondrial DNA (mtDNA) in human immunodeficiency virus (HIV) infected children who face a lifetime exposure to these agents. We performed a cross sectional observation of mtDNA levels in whole blood in a pediatric population to ascertain the relationship between mtDNA, NRTI regimens and parameters of HIV-infection severity.

Methods: Whole blood mt:nDNA ratios were determined by real-time PCR in three groups: 27 presumed HIV-negative, 89 HIV-infected, NRTI-treated and 62 HIV-infected treatment-naive children. Multivariate analysis was used to identify variables independently associated with mtDNA depletion.

Results: Mean mt:nDNA ratios were lower ($P < 0.001$) at 77% of control in the HIV-infected antiretroviral treatment (ART) naïve group and 73% of control in the ART group, but not different between the two HIV-infected groups. Mt:nDNA ratios were negatively associated with age ($P = 0.029$), HIV status ($P < 0.0001$) and Log_{10} of the HIV-1 viral load ($P = 0.035$) and positively associated with CD4 % ($p = 0.032$). A

stavudine vs zidovudine based regimen was associated with lower but not significant levels of mtDNA (P = 0.1).

Conclusions: Depletion of whole blood mtDNA in children is associated independently with HIV-infection and markers of HIV infection severity, and does not improve with either stavudine or zidovudine based ART despite virological control, suggesting that these agents also deplete mtDNA.

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Research Presentations and Grant Awards

The research pertaining to this study or parts thereof have been presented at the following conferences and research days and awarded prizes or grants as delineated.

2004: Awarded MRC and NHLS Trust grant to the value of R70 000.00 as principal investigator for the study – "Mitochondrial:Nuclear DNA Ratios in Peripheral Blood Leukocytes and Lactate in Human Immunodeficiency-virus Infected Children on Antiretroviral Drug Regimens".

2005: George van der Watt presented this work at the Annual Research Day at the School of Child and Adolescent Health on 26th October at Red Cross Childrens' Hospital, Cape Town and was awarded the prize for the best oral presentation by a junior researcher.

2006: This work was selected for a poster presentation at the AACC Annual General Meeting in Chicago, Illinois, USA and George van der Watt was awarded an Earl J Scherago Memorial sponsored International Travel Grant to the value of $2000 for attendance of the meeting.

2006: George van der Watt presented this work at the annual research day of the School of Biomedical Sciences, University of Cape Town and was awarded the prize for the best oral presentation by a MMed student.

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PART A

PROTOCOL

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Research Protocol for the Project Titled

Mitochondrial:Nuclear DNA Ratios in Peripheral Blood Leukocytes and Lactate in Human Immunodeficiency-virus Infected Children on Antiretroviral Drug Regimens

Submitted September 2004

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Note: This is the original title of the proposal submitted for ethics approval and funding. Subsequent to the initiation of the study, the title was appropriately changed to that listed as the title on the title page and part C, the manuscript. Notably peripheral blood leukocytes were changed to whole blood.

1. **Principal Investigator (Applicant)**

George F van der Watt: Division of Chemical Pathology, Red Cross Childrens' Hospital, National Health Laboratory Service and University of Cape Town, MP 049311, george.vanderwatt@uct.ac.za

2. **Project Leader**

Professor Howard Henderson: Division of Chemical Pathology, Red Cross Childrens' Hospital, National Health Laboratory Service and University of Cape Town, MW 003056, howard.henderson@uct.ac.za

3. **Project Title**

Mitochondrial:Nuclear DNA Ratios in Peripheral Blood Leukocytes and Lactate in Human Immunodeficiency-virus Infected Children on Antiretroviral Drug Regimens.

4. **Short Description**

This study will describe the distribution of two potential markers of nucleoside-analogue reverse-transcriptase inhibitor (NRTI) toxicity in human immunodeficiency virus (HIV) infected children, at the HIV Clinic, Red Cross War Memorial Childrens' Hospital, Cape Town, South Africa.

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5. **Clinical Collaborators**

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6. **Supervisors for the degree MMed, Chemical Pathology, University of Cape Town**

Laboratory Supervisor: Professor Howard Henderson

Medical Supervisor: Professor Brian Eley

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7. **Project**

Aim, Objectives and Background

Aim

To determine the distribution of peripheral blood leukocyte mitochondrial:nuclear DNA (mt:nDNA) ratios and venous lactate in paediatric HIV-infected patients prior to and after 6 – 12 months of treatment with various ARV regimens in comparison to controls.

Objectives

- To measure mitochondrial:nuclear DNA (mtDNA:nDNA) ratios in peripheral blood leukocytes (PBLs), and lactate in children with HIV, in comparison to age matched controls.
- To ascertain the effect of HIV infection itself on mt:nDNA ratios in children.
  To ascertain the influence of different HAART regimens on mt:nDNA ratios in children who have received uninterrupted Antiretroviral (ARV) treatment for 6 - 12 months.
- To determine the correlation between serum lactate levels and mt:nDNA ratios in the abovementioned groups.
- To provide information that will ultimately lead to developing awareness of, and a diagnostic and preventative approach to NRTI induced mitochondrial toxicity in children on ARVs in South Africa.

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Background

The use of potent antiretroviral drugs in combination regimens, now referred to as highly active antiretroviral treatment (HAART), has markedly decreased disease progression and mortality rates in HIV-infected patients. NRTIs form the backbone of most currently used ARV regimens used in the treatment of HIV infection (1). Most regimens consist of two NRTIs and either a protease inhibitor (PI) or a non-nucleoside reverse-transcriptase inhibitor (NNRTI). Patients remain on these drug regimens for life, and viral drug resistance and drug toxicity remain problematic (2). Important side effects of HAART in

children are the lipodystrophy syndrome and mitochondrial toxicity (3-5). Decreased bone mineral density and growth retardation are also described (6).

Toxicity of the NRTIs is disadvantageous in the long-term management of viral infection (7). As a group, NRTIs interfere with mitochondrial DNA polymerase gamma, the key enzyme responsible for mitochondrial DNA replication (3,5,8). Stavudine, in particular, has repeatedly been associated with a higher incidence of mitochondrial toxicity than other commonly used NRTIs (9-13). The clinical manifestations of mitochondrial toxicity include: fatigue, weight loss, dyspnoea, nausea and vomiting, abdominal pain, peripheral neuropathy, myopathy, cardiomyopathy, Fanconi syndrome, pancreatitis, hepatic steatosis and death (9,14). Biochemically, mitochondrial dysfunction can present as hyperlactatemia, compensated lactic acidosis or, rarely, as acute decompensated lactic acidosis with a high mortality (4,10).

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In South Africa, availability of ARVs is limited. Many children qualifying for HAART are critically ill and clinical differentiation of NRTI-induced mitochondrial toxicity from HIV-related disease is difficult (Pers comm, B Eley, 2004). Random venous lactate measurements have been suggested as a screening tool for NRTI toxicity in adults and children (11,15,16). Accurate lactate measurements are difficult to obtain in children due to the wide physiological variability associated with physical exertion. Lactate also has a poor specificity for NRTI-induced toxicity (17). Tissue hypoxia, due to various HIV related disease processes is a notable cause of elevated lactate levels.

More recently, investigators have identified measurement of the mitochondrial to nuclear DNA ratio in leukocytes, using various real-time PCR techniques as a direct and more specific marker of NRTI-induced mitochondrial toxicity (10,12,13,15,18,19). These studies have demonstrated a decrease in mitochondrial DNA copy number with NRTI use in adults (10,12,15,18), and an increase in copy number if the NRTI was withdrawn or changed to less toxic NRTIs such as lamivudine. Furthermore, a correlation between low mtDNA copy numbers and symptomatic hyperlactatemia has been demonstrated (10,15). In adults, mtDNA copy numbers decline by a factor of 0.83 log within six months of HAART (13). Furthermore, mean lactate levels have been shown to increase in the first six months of HAART, followed by a plateau without significant change for 3 years (20).

Children are particularly vulnerable to the adverse effects of therapy due to a potentially longer exposure to HAART and because they are growing actively. HIV infection progresses more rapidly in children, and antiretroviral therapy is indicated at an earlier stage than in infected adults. The current Western Cape guidelines recommend that all children with World Health Organisation (WHO) stage II (moderate) and stage III (severe) clinical disease, irrespective of CD4 count, and every child with a CD4 percentage $< 20\%$ (if under 12 months or age) or $< 15\%$ (if ≥ 12 months of age) should receive antiretroviral therapy. These guidelines are in accordance with the recently revised WHO recommendations on antiretroviral therapy in resource-constrained settings (1). Between 70% and 85% of all HIV-infected children managed at Red Cross Childrens' Hospital currently qualify for treatment. Of the 500 - 600 children requiring treatment, some 200 are currently receiving HAART at the Infectious Diseases Clinic. An

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estimated 30-50% have severe disease or advanced immunosuppression. Whether children in the South African setting, with advanced clinical and / or immunological disease are more susceptible to drug toxicity is not known.

HIV infection itself has a marked effect of inducing a measure of mitochondrial dysfunction (21), and asymptomatic, HIV-infected, ARV-naïve adults have significantly lower mtDNA levels than healthy controls (1,13,22). This decrease is greater than that associated with NRTI treatment. Whether the degree of virus induced mitochondrial depletion prior to the onset of treatment influences the risk for mitochondrial toxicity due to NRTIs, remains to be determined. This is a particularly relevant question in South Africa where a large percentage of children who are fortunate enough to receive ARVs have advanced clinical disease.

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By describing the distribution of a prospective marker for HIV/ARV related mitochondrial dysfunction in HIV-infected children, we hope to define the relationship between HIV infection and mt:nDNA ratios in children and the effect of NRTI treatment on these ratios in a paediatric population.

Detailed methodology

Study Population: Patients

Blood samples will be obtained from two separate populations of HIV infected children. The first population will consist of ARV naïve, HIV-infected patients. The second

population will consist of children who have completed 6 – 12 months of uninterrupted HAART and are enrolled in the existing ARV treatment programme operational at the Paediatric HIV Clinic, Red Cross War Memorial Childrens' Hospital. The two populations will be age matched. Written informed consent will be obtained from the legal parents or guardians through an interpreter/councillor in the clinic. Apart from the once off requirement of approximately 0.5ml of blood from each patient, this study will make no additional change to any given patients' routine management as prescribed by the infectious disease consultant. Blood will only be obtained for the study when routine management phlebotomy is performed. Acquisition of samples will continue until at least 100 patient samples have been collected in each group. Drug compliance will be indirectly ascertained as evidenced by adherence to scheduled clinic follow up visits, appropriate return of empty drug containers and response to treatment as determined by measurement of viral-load and CD4 count. Based on current experiences, approximately 90% of patients are expected to be black, Xhosa speaking children residing in the greater Cape Town area of the Western Cape and ± 10% of enrolled patients are expected to default on follow up visits or drug compliance and ± 10% are expected to die within the first six months of HAART (Pers comm, B Eley, 2004). Death in this period is reported to be primarily due to secondary infections or from complications related to immune reconstitution.

Additional data that will be accessed from each patient's routine management information will include the following: demographic data, Centres for Disease Control

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and WHO clinical grading, specific HAART drug regimen prescribed, and routine blood work, which will include viral-loads and CD4 counts.

At present (2004) the preferred first-line regimens for children treated at Red Cross Childrens' Hospital are: Stavudine (or zidovudine) plus lamivudine plus ritonavir for children < 10 kg or < 3 years old, or stavudine (or zidovudine) plus lamivudine plus efavirenz for children > 10 kg or > 3 years of age. Lopinavir / ritonavir co-formulation is used as an alternative to ritonavir or efavirenz in children > 6 months old. Zidovudine is used if patients do not have access to refrigerated storage required for the stavudine suspension.

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Study Population: Controls

A control population of age matched children will be used to establish a normal control group for measurement of PBL mt:nDNA ratios. These children will be sourced from patients within the Division of Paediatric Surgery at Red Cross Hospital who are undergoing elective day surgery for minor procedures. Blood will be obtained from patients who are judged to be clinically free of systemic disease or treatment, that may influence mitochondrial DNA levels or their determination by Real-Time PCR. A 200µl aliquot of EDTA whole blood will only be taken from these children if full prior parental informed consent has been obtained at least 6 hours prior to the elective procedure. In theses cases, parental informed consent will be obtained by the principal investigator. If consent is obtained, the aliquot of blood will only be taken from those children who require an intravenous (I.V) line for the surgical procedure as judged by the attending

anaesthetist. The sample will be collected on placement of the I.V line only. No child will be subjected to a second phlebotomy if sample volumes obtained are inadequate for the purpose of this study. Most I.V lines in this setting are placed after gas induction of light anaesthesia by the attending anaesthetist. In the event of any relative contraindication e.g. time constraints or anaesthetic complications no sample will be taken from that individual patient. To remain within accepted ethical guidelines no child will be tested for HIV infection, nor will any information related to the HIV status of the child, his / her siblings or the mother be elucidated from the child, parent, guardian or caregiver at any stage. Information in regard to prior HIV testing of any of the previously mentioned individuals will not be sought at any stage. Parents or guardians of these patients will only be approached on one occasion to obtain consent. For the purposes of this study these control samples will only be defined as "presumed HIV negative" based on clinical examination and history obtained by the treating physician. For the purpose of this study we aim to obtain between 25 and 50 control samples.

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Laboratory Assays

Peripheral Blood Leukocyte Mitochondrial:Nuclear DNA Ratios

The protocol to be followed is that described by HCF Côte *et al* (10). A 500µl whole blood sample will be collected in acid-citrate-dextrose and kept at 4°C. Required DNA will be extracted from 200 – 300µl of whole blood and purified by means of a Roche High Pure DNA extraction kit within 12 hours of phlebotomy. Extracted DNA will be stored at -70°C until all collections are completed. For each DNA extract, the nuclear

gene for the human polymerase gamma accessory subunit (ASPOLG) and the mitochondrial gene, human cytochrome-*c* oxidase subunit I (CCOI) will be quantified separately by real-time PCR using a Roche Light-Cycler, based in the Division of Virology, University of Cape Town. Reagents will include Roche Light-Cycler Fast-Start DNA Master Hybridization Probe Mix (Roche Applied Science), primers and labelled oligonucleotide probes (Roche Applied Science) and Light-Cycler – Control Kit DNA (Roche Applied Science), for generation of the standard curves. This method allows for expression of the mt:nDNA ratio in PBLs giving a direct count of the average number of mitochondrial DNA copies per nuclear DNA copy per leukocyte.

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Venous Lactate Measurement

Venous lactate measurement will be performed on 15µl of fresh whole blood collected with the use of a tourniquet and avoidance of fist-clenching or lengthy struggling. This blood will be obtained from the same phlebotomy as for the DNA collection and the measurements done at point of care using a point-of-care lactate instrument (BM-Lactate®, Roche Diagnostics GmbH, Mannheim Germany). Collections and measurements will be supervised by the principal investigator. Results will be compared to the reported range of < 2.1mmol/l for venous blood (15,23). This instrument has been correlated with enzymatic wet chemistry lactate methods in adult and paediatric patients.

Statistical Analysis and Study Design

This study is designed as a cross sectional analysis of mt:nDNA ratios in three separate paediatric populations, namely HIV-infected drug naive children, children who have

completed 6 – 12 months of uninterrupted HAART and a control population of clinically non infected children. All three populations will be age matched. The correlation between lactate levels and mt:nDNA ratios in the two HIV infected populations will be assessed statistically with the aim of establishing the value of serial lactate measurements in patients on HAART as an indirect marker of mitochondrial DNA depletion. Lactate levels will not be performed in the control population and a published paediatric reference interval will be used as a comparison. During the collection period, lactate measurements will be performed, and DNA extracted and frozen daily, until sufficient samples have been collected. For mt:nDNA ratios on the light-cycler, control DNA, available as a standard reference material will be used to set up standard curves on the instrument for determination of copy numbers as described by Cote *et al* [9]. Intra and inter-assay coefficients of variation will be calculated for the mt:nDNA assay. Data will be expressed as means and standard deviations (SD) or median and interquartile ranges and means compared using Student's T-test and Student's paired T-test in the case of longitudinal variables. Multivariate analysis models will be built in consultation with a medical biostatistician to search for related variables. Based on the findings of the preliminary data analysis, the HIV infected, drug naïve population will be subdivided according to CDC and WHO clinical criteria and CD4 / viral load counts to determine the correlation between HIV disease severity and mt:nDNA ratios. The drug treated group will also be subdivided by drug regimens to ascertain the effect of different regimens on mt:nDNA. To aid in analysis of mt:nDNA ratios the mean of the control groups will be assumed to represent 100% and the means of the test groups will be expressed as a percentage of the control mean as described by Chiappini F.et al (22).

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Envisaged outputs/outcomes

It is envisaged that this study will provide a clear indication of the distribution of mt:n DNA ratios in children with HIV on HAART in relation to HIV-infected HAART naïve children and controls. This will aid in further defining the effect of HIV infection itself on mitochondrial DNA numbers and provide information on the effect of different drug regimens on this marker. We do not expect lactate levels to correlate well with mt:n DNA ratios in the majority of patients on HAART as elevated lactate is associated with a critical threshold level of mitochondrial dysfunction and has poor specificity. This has been borne out in adult studies. We foresee that this information will be extremely valuable in providing the methodology and groundwork for a longer term, outcomes based study on the effect of HIV induced mitochondrial DNA depletion and subsequent risk for NRTI induced mitochondrial toxicity in South Africa.

Currently, real-time PCR-based assessment of mitochondrial toxicity exists only as a research tool and it is hoped that with a clearer definition of its value it might be developed into a cost-effective and reliable management tool, improving overall patient management and care. We also hope that further research into the long-term effects of HAART in children will be stimulated by this project.

This study will be submitted for journal publication, together with an appropriate conference presentation.

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8. **Impact**

Capacity development

Dr George van der Watt is currently a second year Chemical Pathology registrar (MMed Chem Path) at UCT with the NHLS and will be applying basic research principles throughout the project. He will be setting up a real-time PCR assay for quantitative assessment of human genomic and mitochondrial DNA, currently not available in South Africa, and applying it to diagnostic research. The possibility of training scientists and technologists in the NHLS in the operation of this technique exists, and is relevant to the progressively widening spectrum of applications of real-time PCR.

The clinical-pathological interaction that this study provides between the NHLS Divisions of Chemical Pathology and Virology at Groote Schuur Hosptal and the clinical Division of Infectious Disease at the Institute for Child Health, Red Cross Childrens' Hospital will aid in promoting a more cohesive, integrated approach to paediatric HIV management in our environment.

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9**. Institutional Approval**

This proposal has been approved by the University of Cape Town Research Ethics Committee. Ethics approval reference: 124/2004.

Separate consent forms for each cohort are attached.

10. **Funding**

Source: NHLS Research Trust.

Amount: R 50 000.00 – approved

Source: Department of Infectious Diseases, Red Cross Childrens' Hospital

Amount: R10 000.00-approved

11. **Budget**

Calculated for 250 samples including calibration of instruments

Consumables	Number of units	Unit Cost	Price – VAT inclusive
Primers (nDNA)	1	R747.36	R747.36
Primers (mDNA)	1	R747.36	R747.36
Flourescent probes (nuclear)	2	R11105.10	R11 105.10
Flourescent probes (mito)	2	R11105.10	R11 105.10
Capillaries	1 set	R5253.12	R5 253.12
Conrol kit DNA	1 kit	R2209.32	R2 209.32
Fast Start	3 kits	R2166.00	R6 498.00

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reaction mix			
DNA extraction kit-250	1 kit	R6737.93	R6 737.93
S-lactate NHLS tariff code 4075	250	R58-17	R14 542.50
		TOTAL	R58 945.79

An additional R30 per visit will be provided to all participants in the trial to cover transport costs. This is in keeping with standard practice at the HIV clinic. (R4000.00 – R5000.00).

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PART B

REVIEW

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Literature Review: Mitochondrial Dysfunction and Human Immunodeficiency Virus-infection

Running Head

Mitochondrial Dysfunction and HIV

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Abbreviations

highly active antiretroviral treatment – **HAART**, human immuno-deficiency virus – HIV, nucleoside reverse transcriptase inhibitor – **NRTI**, mitochondrial DNA – **mtDNA**, mitochondrial:nuclear DNA **- mt:nDNA**, mitochondrial DNA – **mtDNA**, didanosine – **ddI**, stavudine - **d4T**, zidovudine – **AZT**, abacavir – **ABC**, tenofovir disoproxil fumarate – **TDF**, emtricitabine – **FTC**, antiretroviral treatment – **ART**, peripheral blood leukocyte – **PBL**, prevention of mother to child transmission **– PMTCT**

Contents

Abstract 141 words; manuscript: 3487 words; 2 figures; 79 references

Abstract

HIV-infection and the pharmacological treatment thereof have both been shown to affect mitochondrial function in a number of tissues and each may cause specific organ pathology through specific mitochondrial pathways. HIV has been shown to kill various tissue cells by activation of mitochondrial apoptosis, and nucleoside analogues, used extensively to treat HIV are known to influence a number of steps affecting mitochondrial DNA integrity. This review describes the basic physiology, pharmacology and pathophysiology of HIV and the nucleoside analogues regarding mitochondrial function, and discusses the progress made in this field with respect to the measurement of these effects and the prediction of potential drug toxicity. The review goes on to highlight areas in the field that require further research and concludes with a summary of the pertinent issues that face us today regarding mitochondrial dysfunction and its relationship to HIV-infection.

Introduction

The success of combination drug highly active antiretroviral therapy (HAART) for advanced human immuno-deficiency virus (HIV) infection in 1996 (1) marked the beginning of an era that would result in HAART becoming the principal regimen of therapy used to treat millions of HIV-infected adults and children around the world. Unfortunately the agents used in these multi-drug regimens are not without side-effects, and nucleoside reverse transcriptase inhibitors (NRTIs), included in most regimens, are responsible for significant morbidity and mortality mostly associated with severe lactic acidosis in patients on HAART (2-7). Nucleoside reverse transcriptase inhibitors were

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already known by the early 1990's, to be mitochondrial toxins causing mitochondrial DNA depletion (8), and since then the toxic effects have been well characterized, less toxic NRTIs have been developed and the risk factors associated with mitochondrial toxicity identified (9). What has however remained elusive is a reliable laboratory marker that can identify pre-clinical mitochondrial toxicity and prevent morbidity. More recently, HIV itself has emerged as a cause of mitochondrial dysfunction, further complicating the diagnosis and management of NRTI associated mitochondrial toxicity. These factors, combined with recent data suggesting that NRTIs, in addition may undermine mitochondrial DNA integrity, has renewed concern over the long term side affects of these agents in patients that could well be exposed to them for life. This review aims to cover the progress of knowledge and provide insight into the mechanisms of HIV associated mitochondrial dysfunction, describing potential implications for patient management, drug development and research in this field.

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Objectives

The objectives of this literature review can be summarized as follows:

1. Describe the literature search strategy used to identify, include and exclude work used to prepare this review.
2. Background: describe the basic physiology of mitochondrial function and how this relates to the pathophysiology of NRTIs and HIV infection regarding mitochondrial function.
3. Describe the history of research in the field of HIV associated mitochondrial toxicity, citing important studies that have contributed significantly towards our

current understanding of the problem and discussing the major strengths and weaknesses of these studies.

4. Identify areas requiring further research.
5. Conclude the review.

1. Literature Search Strategy

Three principal routes were followed to identify and obtain relevant literature cited. Firstly, a number of initial landmark studies consisting of original research and published in high impact-factor journals were identified. The first of these (10) was identified during routine journal review. The references in these papers were then cross referenced and evaluated to identify common papers cited that in turn were evaluated to assess their contribution to the field. This process yielded a core body of essential literature. Secondly the progressive and current literature leading up to the present was regularly searched using the Pubmed electronic database at http://www.ncbi.nlm.nih.gov/pubmed/advanced using a number of search terms and keywords. With each article identified, related articles were searched and reviewed to ensure that new studies were not missed. The third source was directly through personal communication with some authors (10-12) and through reviewers' comments that directly cited important papers when our own work was submitted for publication. Review papers were used where they provided additional data such as study comparisons or provided new guidelines. For the balance, original investigative papers available through Pubmed were used preferentially and where findings overlapped, preference was given to older publications.

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2. Background

Mitochondria are found in all human cells with the exception of mature red blood cells and host a number of essential biochemical reactions largely concerned with fuel oxidation and ATP production (13). In addition, mitochondria are intimately involved in a number of cellular processes including cell differentiation, signaling, and division, and are recognized as being critically involved in programmed cell death or apoptosis and ageing (13-15). Mitochondria also contain their own unique circular DNA (mtDNA), an evolutionary remnant that at 16,5 kilobases, contains 37 genes that code for 13 respiratory chain peptides, 2 rRNA's and 22 tRNA's involved in mitochondrial protein synthesis (16,17). Most mitochondria contain at least 2 copies of mtDNA and the number of mitochondria in cells range from a couple of hundred to hundreds of thousands, in tissues with high energy demands such as muscle, renal tubular cells and neurons (17,18). Mitochondria and their DNA are maternally inherited, derived directly from thousands of maternal oocyte mitochondria. A number of maternally inherited mitochondrial cytopathies with specific mutations in the mitochondrial DNA are well described (17). Two principles govern the manifestation of inherited mitochondrial disease. Firstly the "threshold effect" describes the fact that a critical threshold of dysfunctional mitochondria need to be present before a tissue manifests with mitochondrial failure, due to the high reserve of oxidative capacity inherent in most tissues. Secondly, mitochondria are randomly distributed in the growing zygote in a process known as heteroplasmy. This gives rise to the fact that two patients with identical mtDNA mutations may manifest with different tissue symptoms due to differences in the diseased mitochondrial load in each tissue (17). Mitochondrial DNA is replicated by the enzyme, DNA polymerase gamma,

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this enzyme that performs both polymerase and exonuclease activities differs from the nuclear DNA polymerases in that it is more susceptible to interference by a number of nucleotide analogues designed to inhibit HIV reverse transcriptase (19).

NRTIs and Mitochondrial DNA Integrity

Phosphorylated nucleoside analogues interfere with viral reverse transcriptases by actively competing with endogenous nucleotides for incorporation into the growing nucleic acid chain. After incorporation they prevent chain elongation as they do not contain a 3' hydroxyl group for addition of the next nucleotide (20-22). Examples are shown in Figure 1 (prepared by author).

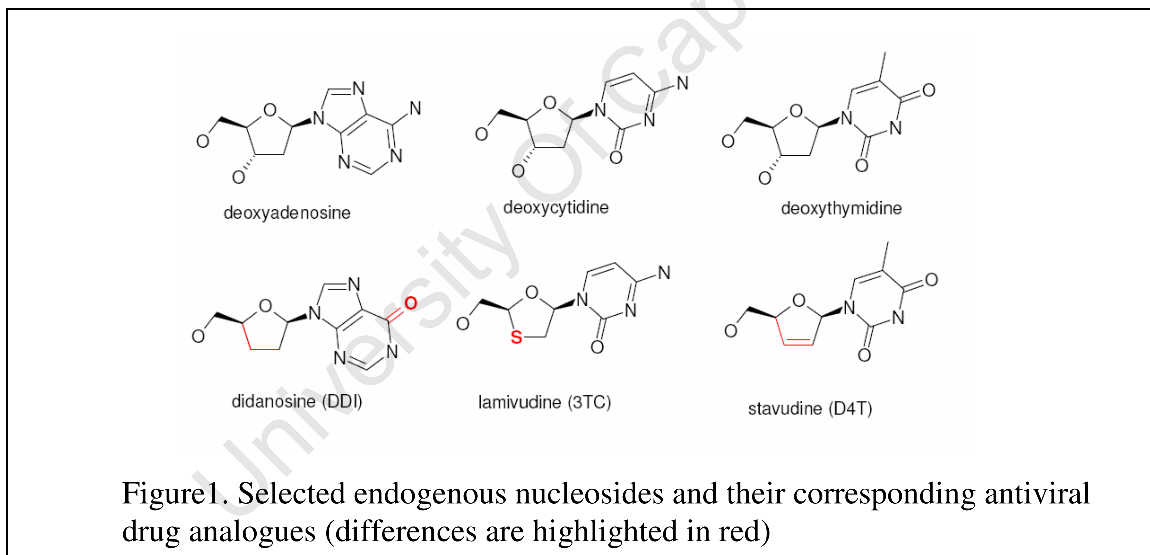

Figure1. Selected endogenous nucleosides and their corresponding antiviral drug analogues (differences are highlighted in red)

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In this manner, NRTIs have been shown to interfere with mitochondrial DNA polymerase gamma in vitro (20), causing depletion of mtDNA that is assumed to result in mitochondrial dysfunction. Major manifestations of this form of toxicity include potentially fatal lactic acidosis, skeletal myopathy, cardiomyopathy, neuropathy, pancreatitis and nephrotoxicity principally manifesting as tubulopathy (2,23-30).

Although all NRTIs affect mtDNA polymerase gamma, abacavir (ABC) together with didanosine (ddI) and stavudine (d4T) have repeatedly been implicated in most severe cases of mitochondrial toxicity with fatal lactic acidosis (10,31,32).

Apart from their direct effect on mtDNA polymerase gamma, nucleoside analogues may also compete with endogenous nucleosides for phosphorylation by mitochondrial thymidine kinase-2 and also for transport into the mitochondria (33,34). Evidence is now emerging that NRTIs may increase the rate of accumulation of mtDNA mutations and increased mutations have been demonstrated longitudinally in peripheral leukocytes in patients on HAART (35). In addition, Maagaard et al found increased mtDNA deletions in skeletal muscle of patients exposed to NRTIs (36) and reduced mitochondrial gene expression has been demonstrated in adipocytes exposed to NRTIs (37,38). Taken together this evidence suggests that NRTIs as a group may have far reaching effects on mitochondrial function in the short as well as long terms. Regarding predisposing risk factors for NRTI mitochondrial toxicity, obese adult females are known to be at increased risk (9,39). Furthermore genetic predisposition linked to the mitochondrial DNA sequence also appears to play a role. Individuals belonging to mitochondrial haplogroup T, that is best characterized as a European mitochondrial lineage have a higher incidence of NRTI associated neuropathy (40), and more recently a known T9098C polymorphism in the mitochondrial gene for adenosine 5'-triphosphate synthase subunit 6 has been associated with severe mitochondrial toxicity in a child after in-utero exposure to AZT and 3TC (41).

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HIV and Mitochondrial Function

HIV causes apoptosis and cellular death in many tissues, most notably in immune cells such as CD4 lymphocytes. It is not surprising therefore that given the importance of mitochondria in regulating cellular apoptosis, that HIV should exploit mitochondrial apoptotic pathways to initiate destruction of immune cells involved in the immune response against infection. Apoptosis is triggered by two major pathways, the first, through exogenous factors that bind cell membrane receptors such as FasL (CD 178) and ultimately give rise to cytoplasmic caspase 8 and 10 activation through a cytoplasmic signaling pathway leading to apoptosis, and the second through intrinsic factors within the cell that trigger cytochrome C and other pro-apoptotic factors to be released from the mitochondrial inter-membrane space, that in turn result in caspase 9 activation and apoptosis (42,43). HIV is implicated in both of these pathways and the mechanisms are illustrated in Figure 2. A number of HIV proteins have been shown directly to activate apoptosis and initiate cell death, the most important of these being Env, Nef, Tat and Vpr (viral protein R). Env, also known as gp-120, together with Nef and Tat have been shown to activate the extrinsic apoptotic pathway through the Fas/FasL receptor(44-46) and Env, Nef, Tat and Vpr have all been shown to trigger cytochrome C release from the mitochondrial inter-membrane space with subsequent caspase activation through the internal pathway. This form of HIV induced apoptosis has been demonstrated in CD4 cells, haematopoietic cells, cardiac myocytes and neurons (47-50). In addition to the direct effect of HIV-1 viral proteins, the massive inflammatory response to HIV and it's associated cytokines including tissue necrosis factor-alpha, interleukin 2 and interferon alpha are also capable of inducing apoptosis (51-53).

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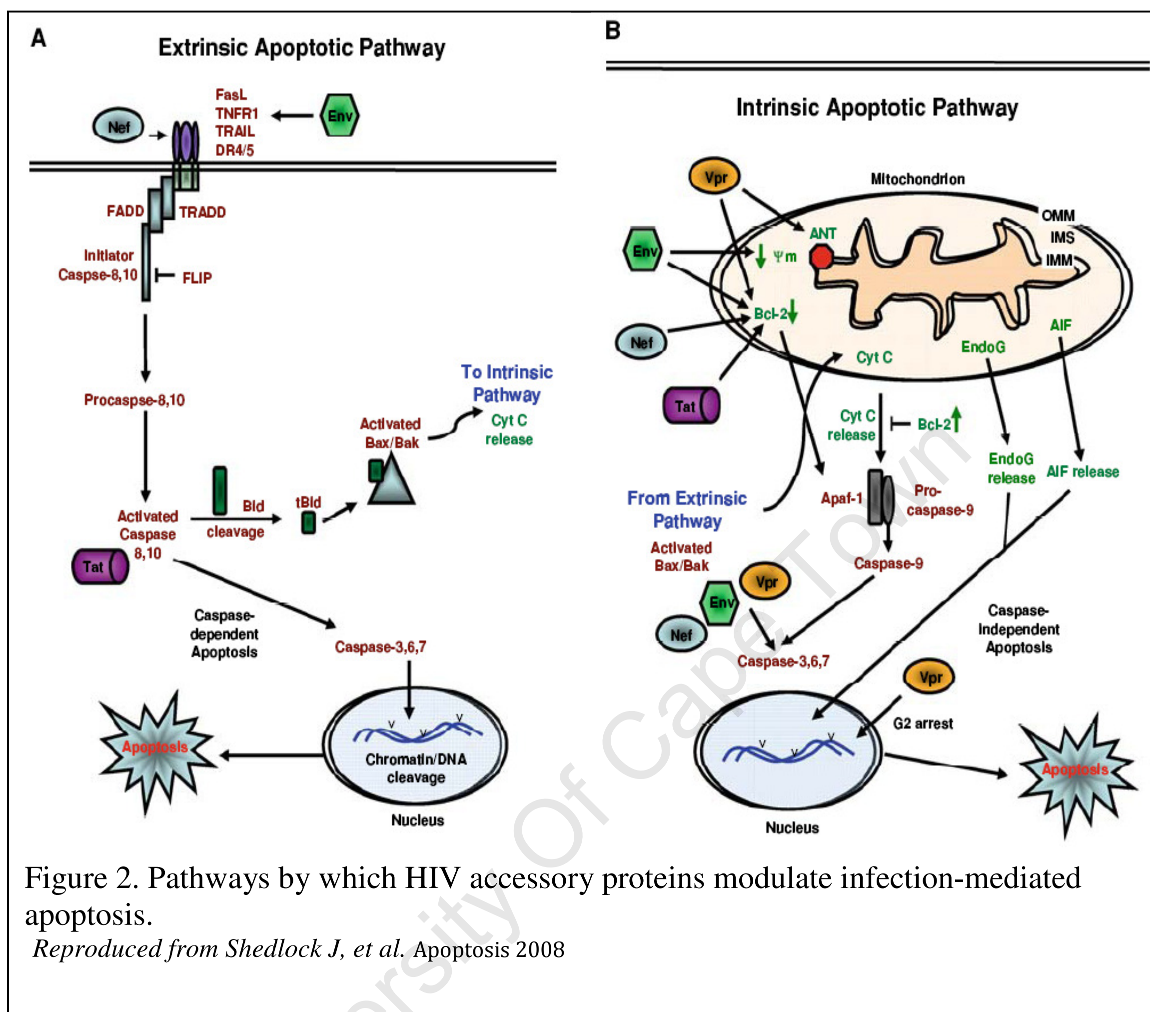

Figure 2. Pathways by which HIV accessory proteins modulate infection-mediated apoptosis.
Reproduced from Shedlock J, et al. Apoptosis 2008

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It is clear from the above mechanisms that both NRTIs and HIV infection itself directly influence mitochondrial function in a number of tissues, HIV by activating mitochondrial apoptotic pathways to trigger cell death and NRTIs by directly or indirectly interfering with mtDNA integrity. It is therefore not surprising that the clinical manifestations of HIV organ pathology and NRTI mitochondrial toxicity should overlap in a number of organs with high dependence on mitochondrial function. Examples include neuropathy, renal tubulopathy, lipodystrophy and myopathy that can be associated with either agent.

3. Research History of NRTI Toxicity (*updated to 2010)*

The mechanism of nucleoside analogue interference with mitochondrial DNA polymerase gamma and the subsequent depletion of mitochondrial DNA were first described in 1990 in early cases of zidovudine (AZT) associated myopathy (8,54,55). In 1995 Lewis and Dalakas put forward their polymerase gamma hypothesis, based on the principle that tissues dependent on high levels of oxidative phosphorylation, where NRTIs were incorporated and phosphorylated were most likely to manifest with mitochondrial dysfunction (56). With the widespread use of dual NRTIs in HAART regimens, and the associated increase in cases of fatal lactic acidosis, researchers looked for markers that could predict mitochondrial toxicity. Blood lactate was the obvious choice and lactate was and is extensively measured and monitored in large cohorts of patients on ART combination regimens. Many of these studies confirmed the increased relative risk of lactataemia with d4T and ddI versus AZT based regimens (57). Lactate measurement however, as a predictive marker of decompensated mitochondrial toxicity was limited by the fact that many adults on NRTI therapy and neonatal recipients of short courses of NRTIs of HIV have asymptomatic hyperlactataemia that does not progress to decompensated lactataemia (58,59). Also lactate lacks specificity and is influenced by technical and physiologic variability (60).

Another approach was to quantify mtDNA against nuclear DNA using real time PCR techniques in peripheral blood leukocytes (PBLs), mainly to avoid invasive tissue biopsies. Most techniques employ separation of a leukocyte buffy-coat from platelets

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because platelets contain mitochondria but not nuclear DNA, which could theoretically confound results. Quantitation is usually performed by simultaneous real-time PCR amplification with fluorometric detection of a mitochondrial and a nuclear gene and the results either expressed as a mitochondrial:nuclearDNA (mt:nDNA ratio) ratio or as absolute mtDNA copies per cell, if separate commercial mtDNA and nuclear DNA standards are used (61,62).This approach yielded promising data in 2002 when in a groundbreaking study, Cotė et al followed 8 HIV-infected hyperlactataemic patients on NRTIs longitudinally. They demonstrated significant depletion of PBL mtDNA in these patients that improved after cessation of therapy to levels comparable to 47 ART naïve HIV-infected patients (10). In addition they reported lower levels of PBL mtDNA in the ART naïve HIV-infected patients versus 24 uninfected controls. The strength of this study was the fact that it was the first to demonstrate the clinical application of PBL mtDNA quantitation as a marker of NRTI mitochondrial toxicity and could be used as a laboratory marker for this purpose but its weakness lay in the fact that the patients selected for longitudinal study all had overt symptomatic hyperlactataemia on NRTI regimens of d4T or d4T/DDI, drugs already known to be associated with increased risk for lactataemia (31,63,64). The study was therefore unable to demonstrate whether PBL mtDNA could be used to detect pre-symptomatic mitochondrial toxicity before the onset of clinical lactataemia or improve on the specificity or sensitivity of lactate as a marker of mitochondrial toxicity. Subsequent to this work a number of studies were conducted to evaluate PBL mtDNA in HIV cohorts to answer these questions (12,37,62,65-69). Five of these studies confirmed the findings of Cotė et al, namely that PBL mtDNA levels in ART naïve HIV-infected patients were depleted versus HIV-negative control groups but

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not significantly different from ART-exposed patients. Some conflict however existed in the small (n=10) cross sectional study by Henry et al (65) that demonstrated no difference between HAART exposed patients and healthy controls and in the longitudinal study of Miura et al (67) that demonstrated complete amelioration of PBL mtDNA depletion to normal levels with AZT/3TC based HAART, suggesting that HIV was the major cause of depleted PBL mtDNA. These findings have more recently been supported by Aldrovandi et al who measured PBL mtDNA in babies exposed to NRTIs in utero and as neonates. They found that PBL mtDNA was decreased in HIV exposed neonates compared to controls but higher in ART/HIV exposed neonates than HIV exposed neonates who were not exposed to ART (70). These data suggest that HIV itself is the major contributor towards PBL mtDNA depletion and not NRTIs. Regarding ART exposed patients in larger studies, Chiappini (12), Cotė (68), and De Mendoza et al (66) all found lower levels of PBL mtDNA associated with d4T, DDI and particularly d4T/DDI combination regimens. In the largest of these, a cross sectional study by Cotė et al (68) , 214 ART treated individuals exposed to ART for > 4 months clearly demonstrated progressive PBL mtDNA depletion with AZT > d4T > ddI > d4T/ddI combination. Today, both d4T and ddI are not recommended for first line HAART regimens and are being replaced by safer NRTI combination regimens due to their association with mitochondrial toxicity. Stavudine is currently only used as a first line drug in resource constrained settings due to its lower cost. Paediatric HIV presents additional scenarios where children who do not contract HIV are exposed to ART either in utero, during maternal treatment or perinatally during PMTCT of HIV. Mitochondrial toxicity associated with PMTCT was highlighted in 1999 when 8 HIV-uninfected children in a French cohort exposed to AZT and/or 3TC

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presented with severe mitochondrial dysfunction, 2 of whom died (71). Subsequent prospective cohort studies have been conflicting however, with some reporting evidence for an increased incidence of mitochondrial dysfunction up to 18 months post PMTCT (72), and others not (73,74). Despite this, transient hyperlactataemia that rarely may progress to fulminant lactic acidosis in ART exposed neonates is well known (58,59). Based on the outcomes of the South African arm of the Children with HIV Early Antiretroviral Therapy (CHER) study (75), treatment guidelines for paediatric HIV have been changed and now include ART for all HIV infected children under 12 months of age regardless of clinical condition (76). In addition, a move to reduce the use of d4T in resource constrained settings is being advocated with substitution of d4T with TDF in adults and ABC in children as one of the first line NRTIs (76).

Due to the conflicting findings with PBL mtDNA measurement, researchers turned their attention to tissue mtDNA quantitation. Mitochondrial DNA measured by Cotė et al (77) in renal biopsies of patients without HIV, with HIV plus tenofovir (TDF) and with HIV but without TDF demonstrated reduced mtDNA levels in both HIV infected groups versus uninfected biopsies but no difference between the two HIV groups, suggesting that HIV itself also contributed toward mtDNA depletion in renal tissue. Buffet et al (78) measured adipose tissue mtDNA in a large cohort of HIV infected individuals with lipodystrophy on HAART and found depleted mtDNA levels versus controls and a significant association between d4T and ddI and mtDNA depletion. The contribution of this study was limited by the fact that only 2 HIV-infected ART naïve patients were studied and so the contribution of HIV to adipose tissue mtDNA depletion was not assessed (78). The value of tissue mtDNA measurement has been questioned by Kim et al

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(79), who confirmed Buffets findings of depleted mtDNA in adipose tissue of lipoatrophic HAART treated patients but found no decrease in mtDNA dependent mitochondrial function and an actual compensatory increase in nuclear driven mitochondrial biogenesis, suggesting that mtDNA depletion was not a good marker for mitochondrial function. In a cross sectional study by Magaard et al (36), mitochondrial DNA was lower in muscle biopsies from 24 patients with HIV on HAART than 10 ART naïve HIV patients, but both groups demonstrated decreased PBL mtDNA compared to 11 healthy controls They concluded that PBL mtDNA does not correlate with organ mtDNA depletion. Taken together, the measurement of mtDNA in end organs appears to deliver similar information to PBL measurements, namely that NRTIs, particularly d4T and ddI cause tissue mtDNA depletion, HIV infection may cause depletion independently but this is likely to be a tissue dependent phenomenon, and mtDNA levels in tissue do not correlate with PBL mtDNA levels or with actual mitochondrial function due to the initiation of compensatory mechanisms to preserve essential mitochondrial functions. Currently most authors have concluded that measurement of mtDNA in PBLs and tissue contribute little toward predicting NRTI induced functional mitochondrial toxicity and mtDNA measurements are not used in routine practice though there may still be some value in performing serial measurements of PBL mtDNA in at-risk individuals. Due to the technical difficulties in obtaining representative, functional mitochondrial specimens, simple assays for assessing mitochondrial function are not available and for the most part, clinicians are still left with good clinical judgment and lactate measurements as the only way to detect early NRTI associated decompensated lactataemia, the most serious side effect of these agents. Subsequent to these findings, more recent work has focused on the

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influence of HIV itself on mitochondrial function, largely to explain the mechanisms of HIV induced immunosuppression and organ pathology as discussed above.

4. Further Research

No study to date has managed to answer a number of pressing questions regarding the combination effect of HIV and NRTIs on mitochondrial function. A number of these questions can only be answered in longitudinal studies and some are particularly applicable to children. Research questions that need to be answered are the following:

- What are the long term complications of NRTIs, specifically regarding mitochondrial DNA integrity and its effect on ageing and end organs with high energy requirements and to what extent are tissues able to compensate for NRTI induced mtDNA depletion? This question is especially relevant to children who will now be exposed these agents for life with typical onset soon after birth.
- Are the mitochondrial effects of HIV and NRTIs synergistic – in other words are patients with severe HIV disease at a higher risk for mitochondrial toxicity from NRTIs than patients with less severe HIV disease, and if so which tissues are most likely to be affected? This question is especially relevant in resource constrained settings where access to ART is limited and many patients have more severe disease prior to initiation of treatment with regimens often containing d4T.
- Whilst safer, cost effective ART is developed, is there another laboratory marker or tissue specific marker that can be used to identify patients at high risk for

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NRTI toxicity preemptively so that preventative management can be employed in time?

5. Conclusion

The availability of HAART has made an enormous contribution worldwide to people living with HIV and the focus has now shifted to the prevention of long term side effects of the drugs used in treatment of this disease. Whilst lipodystrophy and its associated metabolic changes are associated with protease inhibitors, the major toxic effect of NRTIs is fatal decompensated lactic acidosis due to mitochondrial toxicity. There is also growing concern about the cumulative long-term effects of a class of drugs that influence mtDNA synthesis and repair.

Although the availability of safer ART regimens has reduced the incidence of severe toxicity, new drugs are often not available in resource constrained settings where the majority of people with HIV find themselves, and though measures to limit NRTI toxicity such as regimen switching are available, there is a pressing need to implement protocols that promote the use of safer first-line NRTIs and restrict the use of d4T. It is also crucial to continue further research into the understanding and prevention of ART related toxicity, particularly as it relates to children with HIV.

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PART C

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Title

Whole Blood Mitochondrial DNA Depletion in Human Immunodeficiency-virus Infected Children

Running Head

Mitochondrial DNA Depletion in Paediatric HIV

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Abbreviations

peripheral blood leukocyte – **PBL**, mitochondrial:nuclear DNA **- mt:nDNA**, nucleoside reverse transcriptase inhibitor – **NRTI**, mitochondrial DNA – **mtDNA,** antiretroviral treatment – **ART**, highly active antiretroviral treatment – **HAART**, stavudine - **d4T** zidovudine – **AZT**, Didanosine – **DDI**, lamivudine - **3TC**, ritonavir – **RIT**, efavirenz – **EFV**, lopinavir – **LPV**, prevention of mother to child transmission – **PMTCT**

Contents

Abstract 249 words; manuscript 2733 words; figures 3; tables 3; references 40.

Abstract

Background: Nucleoside reverse transcriptase inhibitors (NRTIs) affect mitochondrial DNA polymerase gamma causing significant toxic effects, including fatal lactic acidosis. Little is known about mitochondrial DNA (mtDNA) in human immunodeficiency virus (HIV) infected children who face a lifetime exposure to these agents. We performed a cross sectional observation of mtDNA levels in whole blood in a pediatric population to ascertain the relationship between mtDNA, NRTI regimens and parameters of HIV-infection severity.

Methods: Whole blood mt:nDNA ratios were determined by real-time PCR in three groups: 27 presumed HIV-negative, 89 HIV-infected, NRTI-treated and 62 HIV-infected treatment-naive children. Multivariate analysis was used to identify variables independently associated with mtDNA depletion.

Results: Mean mt:nDNA ratios were lower ($P < 0.001$) at 77% of control in the HIV-infected antiretroviral treatment (ART) naïve group and 73% of control in the ART group, but not different between the two HIV-infected groups. Mt:nDNA ratios were negatively associated with age ($P = 0.029$), HIV status ($P < 0.0001$) and Log_{10} of the HIV-1 viral load ($P = 0.035$) and positively associated with CD4 % ($P = 0.032$). A stavudine V.S zidovudine based regimen was associated with lower but not significant levels of mtDNA ($P = 0.08$).

Conclusions: Depletion of whole blood mtDNA in children is associated independently with HIV-infection and markers of HIV infection severity, and does not improve with either stavudine or zidovudine based ART despite virological control, suggesting that

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these agents also deplete mtDNA. The long-term consequences of this potentially toxic effect remain a cause for concern

Introduction

Nucleoside reverse transcriptase inhibitors form the backbone of all current first line ART regimens used to treat paediatric HIV in South Africa. Patients remain on these drugs for life, and viral drug resistance and drug toxicity remain problematic. Major side effects of NRTIs are the lipodystrophy syndrome and potentially fatal lactic acidosis, in addition to organ effects such as myopathy, cardiomyopathy, neuropathy, pancreatitis and nephrotoxicity (1-9).

Mitochondrial toxicity associated with NRTIs as a group is thought to be principally due to interference with mitochondrial DNA polymerase gamma, the enzyme responsible for mitochondrial DNA replication with resultant depletion in mtDNA (10-12). Stavudine (d4T) , a widely used NRTI in cost constrained settings, has repeatedly been implicated in cases of mitochondrial toxicity (13-15). Other mechanisms thought to contribute to toxicity of these agents include inhibition of mitochondrial thymidine kinase-2 and competition between endogenous nucleotides and phosphorylated NRTIs for transport into the mitochondria (16,17). There is now evidence to suggest that these drugs may affect the integrity of the mitochondrial genome and not just mtDNA quantity. Nucleoside reverse transcriptase inhibitors have been linked to accumulation of mtDNA mutations in peripheral blood leukocytes (18) and mtDNA deletions in muscle (19).

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Decreased expression of mitochondrial genes has also been demonstrated in adipocytes exposed to NRTIs even in the absence of reduced mtDNA (20,21).

HIV infection itself also appears to contribute towards mitochondrial dysfunction and a number of initial studies looking at NRTI toxicity demonstrated peripheral blood leukocyte (PBL) mtDNA depletion in treatment naïve individuals. This has been supported by the fact that reduced mtDNA content, decreased oxidative phosphorylation activity, and reduced mitochondrial membrane potential have all been found in ART-naïve patients with HIV, mainly in PBLs (22-28). In addition, evidence of organ mitochondrial derangement has been found in skeletal muscle, fat and renal tissue in treatment naïve individuals (28-31). The mechanisms underlying HIV's effect on mitochondria are less well understood but a number of cytokines associated with persistent immune activation together with directly acting viral proteins are thought to induce mitochondrial apoptosis with end point reduction in mtDNA and mitochondrial function (32,33).

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The World Health Organization recommendations on ART in resource-constrained settings were followed at our institution during enrolment for the study and between 70% and 85% of all HIV-infected children managed at Red Cross Childrens Hospital qualified for treatment (34) (Pers comm. B Eley). This however is set to increase with the implementation of new guidelines based on data from the South African arm of the Children with HIV early antiretroviral therapy (CHER) study (35), that recommends ART for all HIV-infected infants under 12 months of age (36). Of the children who

required treatment, about 30% were receiving highly active antiretroviral treatment (HAART) at the Infectious Diseases Clinic and 30-50% had severe disease or advanced immunosuppression (Pers comm. B Eley). Children are theoretically more vulnerable to the adverse effects of NRTIs due to a potentially longer exposure and because they are growing actively and have higher energy demands. Also, HIV-infection progresses more rapidly in children, and ART is indicated at an earlier stage than in adults (37). Many children who do not contract HIV are still exposed to ART in utero during maternal treatment or perinatally during prevention of mother to child transmission (PMTCT) of HIV. Mitochondrial toxicity associated with PMTCT with AZT and/or 3TC has been linked to severe mitochondrial dysfunction (38), but subsequent prospective cohort studies have been conflicting, with some reporting evidence for an increased incidence of mitochondrial dysfunction after PMTCT (39), and others not (40,41). Despite this, transient hyperlactataemia that rarely can progress to fulminant lactic acidosis in ART exposed neonates is well described (42,43). The effect of HIV and NRTIs on mitochondrial function in children remains poorly understood, the most important questions being, whether children with advanced immunological disease are more susceptible to drug toxicity or not and what the potential long term effects of NRTIs on mitochondrial genome integrity and gene expression are and how these effects could manifest over a lifetime exposure to these drugs (44). These are particularly relevant questions in South Africa where many children fortunate enough to receive ART have advanced clinical disease and many will remain on NRTIs such as d4T for the foreseeable future.

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In South Africa ART availability is limited. Many children qualifying for ART are critically ill and clinical differentiation of NRTI-induced mitochondrial toxicity from HIV-related disease is difficult. Venous lactate measurements have been suggested to screen for NRTI toxicity in adults and children (14,15,45), but representative lactate measurements are difficult to obtain. Lactate also has a poor specificity for NRTI toxicity.

Quantitation of mtDNA in PBLs, using real-time PCR has been investigated in adults as a marker of mitochondrial toxicity (13,22,23,27,46). In the first of these studies (13), PBL mtDNA depletion was associated with symptomatic hyperlactataemia in 8 adults taking d4T. In all patients, mtDNA recovered after interruption of therapy. Three other subsequent cross sectional studies have demonstrated similar findings and shown more pronounced PBL mtDNA depletion with the use of d4T and didanosine (ddI) based regimens together with lower levels of PBL mtDNA in treatment naïve infected adults compared to healthy controls (22-24).

The objectives of this study were to describe the distribution of whole blood mtDNA, as a marker of mtDNA depletion in children, and to assess the effect of NRTI treatment on this parameter. We also sought to determine the relationship, if any, between whole blood mtDNA and lactate as well as other markers of HIV-infection severity. To our knowledge these parameters have not been assessed in a pediatric population.

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Materials and Methods

Setting

HIV-infected children attending the outpatient HIV Clinic under the Division of Infectious Diseases at the Childrens Red Cross War Memorial Hospital and presumed HIV-negative children attending outpatient surgery at the same institution were enrolled into the study between October 2004 and March 2005. Study approval was obtained from the Research Ethics Committee of the University of Cape Town. Ethics approval reference: 124/2004.

Patients

Sixty-two (62) ART-naïve HIV-infected children aged 3.4 – 121.3 months, 89 ART exposed children aged 10.2 - 130 months, and 27 presumed HIV-negative children aged 10.5 – 113 months were included in the cross sectional analysis. Children were assigned to the HIV-infected ART-naïve group if they were due for routine management phlebotomy, were HIV positive and had never been exposed to any ART regimen other than PMTCT. HIV-infected ART-exposed children were included if they had completed more than 6 months of uninterrupted single regimen ART and if routine management phlebotomy had been requested on the day of their clinic visit. Children attending minor outpatient surgery were enrolled in the control group and presumed HIV negative by way of no prior physician diagnosis of HIV. Their HIV status was not requested nor tested on ethical grounds.

In the ART-exposed HIV-infected group, 22 children had received a zidovudine/lamivudine (AZT/3TC) based regimen with either ritonavir (RIT) (n=12), efavirenz (EFV) (n=4) or lopinavir/ritonavir (LPV/RIT) (n=6) constituting the 3rd drug,

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67 children had received a d4T/3TC based regimen with either RIT (n=23), EFV (n=38), LPV/RIT (n=4) or nevirapine (n=2).

All children were accompanied by a parent or legal guardian on the day of visitation and informed consent was obtained according to the rules of our institution. Age, sex, weight and height data were obtained from all children. CD4 count, HIV-1 viral load and type and duration of treatment data were obtained for all ART exposed children. CD4 count was obtained for all ARV-naïve HIV-infected children and HIV-1 viral load was obtained in 23 patients in this group.

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Blood sampling and data collection

Anthropometric data: Age, sex, height and weight data were analyzed using Epi Info™ (2004/09/07) software based on the CDC/WHO 1978 reference to estimate weight for height Z-scores.

Blood sampling: Children were restrained, when necessary by an assistant during routine phlebotomy and free flowing venous blood was collected without the use of a tourniquet. A phlebotomy was arbitrarily classified as difficult if more than 1 minute of crying or struggling against restraint occurred before blood was obtained. In the control group, blood was obtained at the placement of a routine management intravenous catheter after induction of general anesthesia.

Lactate: Lactate was measured on whole blood using a portable, lactate analyzer (BM-Lactate®, Roche Diagnostics GmbH, Mannheim Germany) by enzymatic reflectance photometry. The instrument correlates with reference methods (47) and quality control

was performed daily using control solutions. Values > 2.1mM were considered to be elevated (15,42). Lactate was not measured in the control group as their phlebotomy conditions were considered non-representative.

HIV-1 Viral load and CD4 count: Leukocytes for CD4 counts were prepared using ImmunoPrep™ Reagent System, CD4 and CD45 cells were labeled using FlowCare™PLG CD4 Reagent and CD4 counts determined on a Beckman Coulter® Epics-XL-MCL flow-cytometer (Beckman Coulter Ireland, Inc).RNA for HIV-1 viral loads was extracted by a Nuclisens® easyMag™ system and viral load determined using a Nuclisens® Easy-Q-HIV-1 v1.1 analyzer (bioMérieux bv, NL-5281, RM Baxtel, The Netherlands).

Quantitation of Mitochondrial DNA by Real-Time PCR

DNA Preparation: 0.5mL blood collected into K-EDTA BD Vacutainer ® tubes was stored at room temperature until same-day DNA extraction. Total DNA was extracted from 200µL whole blood using a Roche High Pure PCR Template Preparation kit (Roche Diagnostics) into 200µL elution buffer. Extracted DNA was stored at -20Ċ until assayed.

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MtDNA quantitation: mtDNA was quantified using an in-house method based on that described by Cŏtė et al (13). Briefly, small regions of the nuclear gene for human polymerase gamma 2 accessory subunit (ASPOLG-2), and the mitochondrial gene human cytochrome-c oxidase subunit 1 (COX-1) were quantified separately by real-time quantitative PCR on a Roche Light Cycler. This system utilises fluorescence resonance energy transfer technology with 3'fluorescein-labeled and 5' LC-Red 640-labeled

oligonucleotide hybridization probes to quantify the PCR reaction. Control DNA prepared from pooled DNA from each of the 27 controls, was used to set up both the nuclear and mitochondrial gene standard curves and the mt:nDNA ratio was arbitrarily set at 1. All PCR reactions were performed in duplicate. Inter-assay coefficients of variation for this assay, derived from 2 patient controls repeated in 19 consecutive runs over 5 days were 5.1% for the mitochondrial gene, 7.4% for the nuclear gene and 8.5% for the mt:nDNA ratio, respectively.

Statistical analysis

Results are expressed as means and standard deviations (SD) or median and interquartile range. Means were compared with Student's T-test and Student's paired T-test in the case of longitudinal variables. Type I error was set at 5%. Multivariate analysis models were built using Stata version 8.0 software with statistical uncertainty estimated from 95% confidence intervals.

Results

Fundamental study group characteristics are depicted in Table 1 (p73) and laboratory parameters compared in Figure 1 (p74). Children in the ART group received HAART for a median of 11.8 months with 64% demonstrable suppression of viral replication (HIV-1 viral load < 400 copies/mL). As expected the efficacy of ART was further evidenced by the fact that treated children had significantly higher CD4 percentage counts and improved weight for height Z scores compared to ART-naïve HIV-infected children. Mean lactate levels were not significantly different between untreated (2.1 $\pm$ 0.6 mmol/L

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and treated HIV-infected patients (2.1 ± 0.5 mmol/L). As expected, difficult phlebotomy was associated with elevated lactate in children from both groups (2.5 ± 0.5mmol/L, $P < 0.0001$). No cases of symptomatic lactic acidosis or lactate > 5mmol/L were encountered.

Mitochondrial:nuclearDNA ratios between study groups are depicted in Figure 2 (p75). The mean mt:nDNA ratio in presumed HIV-negative control group (n = 27) was 1.13 ± 0.22. If this value was assumed to represent 100%, then the mt:nDNA ratio in the ARV-naïve HIV-infected group (n = 62) was 77% of control (0.87 ± 0.3, $P < 0.0001$) and 73% of control (0.82 ± 0.23, p < 0.0001) in the ART-treated HIV-infected group (n=89) respectively. Although the mean ratio was lower in the ART treated group, there was no significant difference in mt:nDNA ratios between ARV-naïve and ART treated HIV-infected children ($P = 0.242$). In the ART treatment group, depicted in Figure 3, a d4T based regimen was associated with a lower, but not significant level of mtDNA ($P = 0.08$) when compared to an AZT based regimen.

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Multivariate analysis

Multivariate analysis of data obtained from HIV-infected children is depicted in Table 2 (p77). As expected from prior data, mt:nDNA ratios were independently negatively associated with HIV status (p < 0.001) in all analyses and no association could be found between mt:nDNA ratios and NRTI treatment as a whole, absolute CD4 counts or lactate levels.

In the model looking specifically at children with HIV on ART depicted in Table 3 (p78), there was a significant negative association between mitochondrial DNA depletion and age ($P = 0.018$) and the Log_{10} of the HIV-1 viral load ($P = 0.044$), furthermore this model

demonstrated a significant positive association between CD4 percentage of total lymphocyte count (*P* = 0.032) and mitochondrial DNA. We were concerned that the increase in mtDNA seen with effective viral control might be a proxy measure of increased lymphocytes as a percentage of total leukocytes and for this reason we deliberately included this parameter to correct for this possible confounding effect. Multivariate analysis in this group again confirmed lower mtDNA levels associated with the use of a d4T based regimen.

Discussion

The data, derived from a large cohort of subjects demonstrates that mtDNA is significantly depleted in whole blood of ART-naïve HIV-infected children as measured by real-time PCR and that this depletion was present before institution of ART. Depletion of mtDNA is associated independently with HIV-infection and correlated with markers of infection severity, namely increasing HIV-1 viral load and decreasing CD4 percentage and is not influenced by total lymphocyte depletion. Furthermore mtDNA levels did not change significantly after a median of 11.8 months of HAART, despite good evidence for successful suppression of viral replication and clinical improvement. Stavudine based regimens were associated with lower mtDNA levels compared to AZT based regimens but the difference in children did not approach significance. No cases of clinically suspected NRTI toxicity were detected during the study.

A number of limitations were present in the study. Firstly, mt:nDNA ratios were determined in whole blood containing platelet mitochondria together with leukocyte

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nuclear and mitochondrial DNA and secondly, HIV status was not confirmed in the control group. Both factors could potentially increase the distributions obtained for mtDNA in each group, reducing the number of statistical associations. In this study, sample volumes were too small to effectively separate a buffy coat for PBL DNA extraction and so whole blood was used. In addition, a published reference interval for lactate was used resulting in an abnormally high percentage of hyperlactataemia or suggesting poor phlebotomy technique. This stresses the requirement for in-house reference ranges, particularly for lactate in a paediatric setting and highlights problems associated with extrapolating published reference intervals and cutoffs.

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In agreement with adult based studies, this work has demonstrated that mtDNA depletion is present in children with HIV and that this depletion is dependent on HIV-infection severity and does not improve with ART containing NRTIs. This depletion most likely has a dual etiology. Firstly due to the known inhibitory effect of NRTIs on mitochondrial DNA polymerase gamma and secondly, that associated with HIV-infection itself. How the mitochondrial effects of NRTIs, particularly d4T, which is still widely used in South Africa, will manifest in the long term remains to be seen and longitudinal studies in children are needed in this regard. Due to the fact that both NRTIs and HIV may influence the level of whole blood mtDNA, the value of this, or other blood based mtDNA quantification markers to predict or diagnose life threatening NRTI toxicity with lactic acidosis is doubtful. Regular clinical assessment may still be the most viable way to prevent drug related fatalities together with the implementation of regimens that employ safer first-line NRTIs and limit the use of d4T (36). This is especially relevant to

resource constrained settings, where the majority of children who face a lifetime exposure to these drugs are to be found.

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Table 1: Fundamental Study Group Characteristics

	Controls, presumed HIV negative	**HIV-infected ART naïve**	**HIV-infected on ART**
n	27	62	89
Age in months Median Interquartile range	 58 36 – 73	 36 17 - 77	 33 19 - 62
Weight for height Z-score Median Interquartile range	 -0.44 -0.97 - +0.34	 -0.87 -1.47 - -0.03	 0.28 -0.42 - +0.90
Gender Proportion male	 48%	 42%	 56%
CD4% of total lymphocyte count Mean SD	 N/A	 15.3 8.7	 23.3 9.2
Duration of HAART in months Median Interquartile range	 N/A	 N/A	 11.8 6.3 – 17.4
HIV-1 viral load < 400 copies/mL, n (%)	N/A	N/A	56 (64%)
NRTI exposure – *n* (%) d4T/3TC AZT/3TC	 N/A N/A	 N/A N/A	 67 (75%) 22 (25%)

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Figure 1: Comparative laboratory parameters between ART (n=89) and non-ART (n=66) groups of HIV-infected children. Box-and-whisker plots: whiskers = non-outlier range, box = 25th to the 75th percentiles, marker = median; *** denotes $P <$ 0.0001

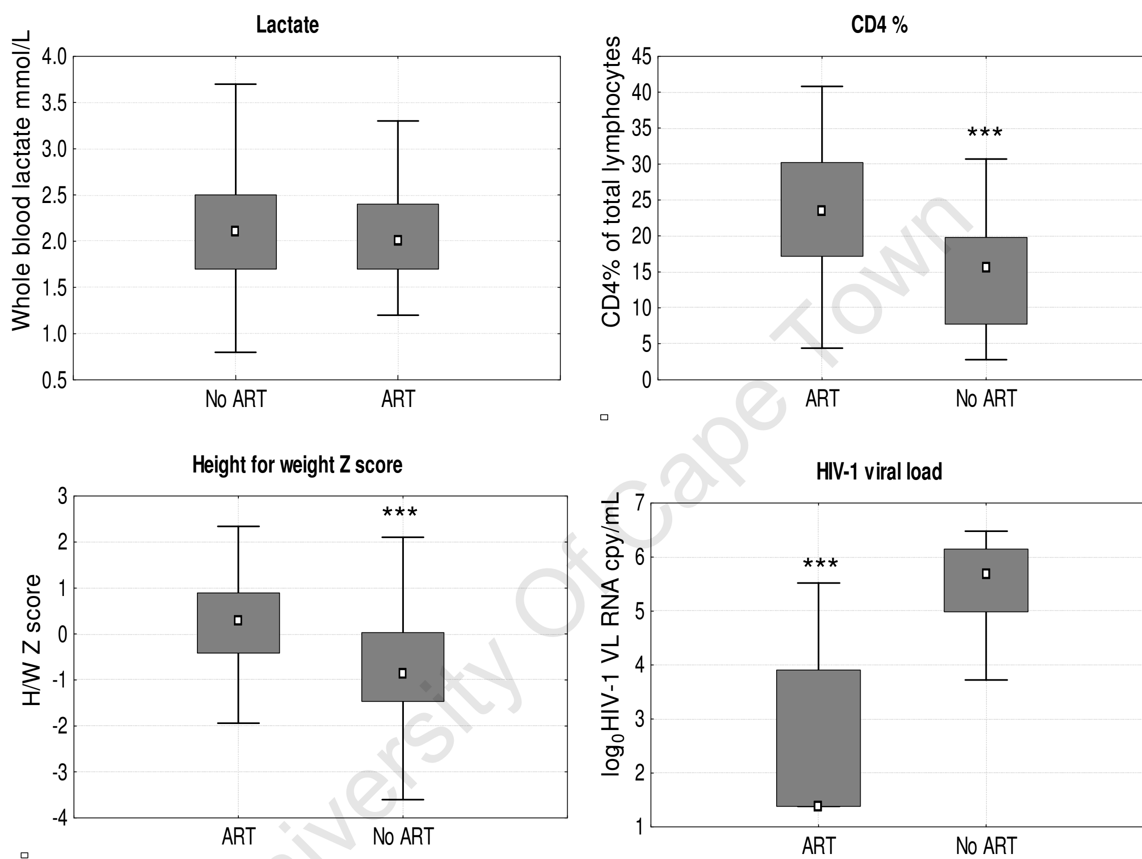

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Figure 2: Mitochondrial:nuclearDNA ratios between study groups. Box-and-whisker plots: whiskers = non-outlier range, box = 25th to the 75th percentiles and the marker = median (50th centile); *** denotes $P < 0.001$, compared to control.

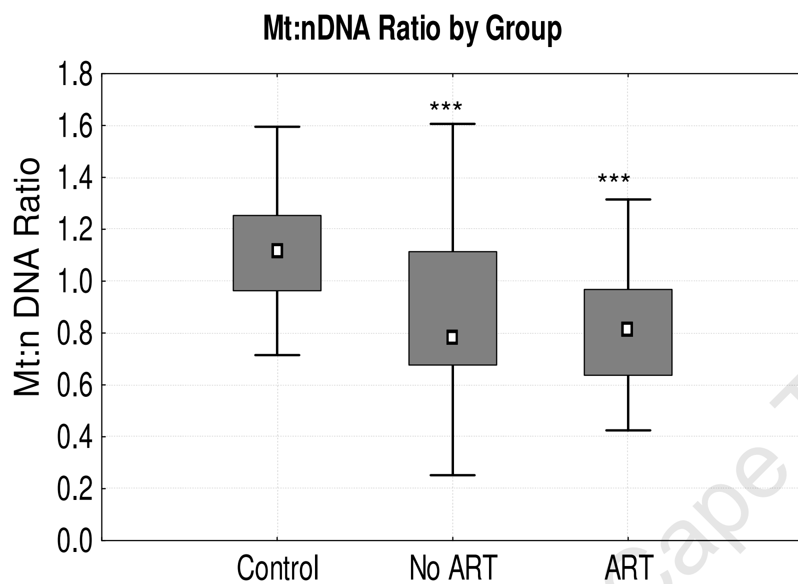

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Figure 3: Mitochondrial:nuclearDNA ratios between children treated with d4T VS an AZT based regimen. Box-and-whisker plots: whiskers = non-outlier range, box = 25th to the 75th percentiles and the marker = median (50th centile); *P* denoted on the plot.

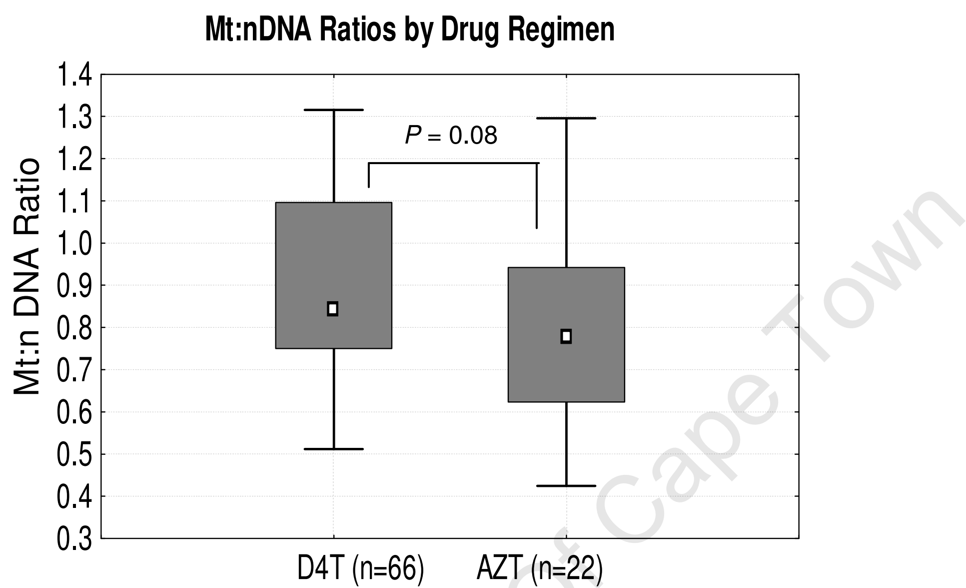

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Table 2: Multivariate analysis data assessing impact of HIV on Mitochondrial:Nuclear DNA ratio, adjusted for co-variates (n = 172)

Attribute	**Co-efficient**	**95% CI**	**p-value**
HIV-infected	-0.26	(-0.38 – -0.14)	<0.001
Female sex	0.07	(-0.01 – 0.15)	0.079
Per-year increase in age	-0.01	(-0.03 – 0.00)	0.084
Per-unit increase in weight for height Z-score	0.02	(-0.01 – 0.05)	0.144
On HAART	-0.07	(-0.16 – 0.02)	0.143
Constant	1.17	(1.04 – 1.29)	

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Table 3: Multivariate analysis data assessing predictors of Mitochondrial:Nuclear DNA ratio in HIV-infected patients on HAART, adjusted for co-variates (n = 80)

Attribute	Co-efficient	95% CI	p-value
Female sex	0.03	(-0.07 – 0.12)	0.586
Per-year increase in age	-0.03	(-0.05 – 0.00)	0.018
Per year treatment duration	0.002	(-0.06 – 0.062)	0.928
d4T based regimen	-0.09	(-0.19 – 0.01)	0.084
Per-unit increase in weight for height Z-score	0.01	(-0.02 – 0.04)	0.517
Log10 most recent HIV-1 viral load	-0.04	(-0.08 – 0.00)	0.044
Per 10% increase in CD4% of total lymphocyte count	0.07	(0.01 – 0.13)	0.032
Per 10% increase in lymphocyte% of white cell count	0.00	(-0.03 – 0.04)	0.787
Constant	0.83	(0.50 – 1.17)	

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PART D

SUPPORTING DOCUMENTS

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Data Capture Forms

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Data Capture Form: ARV Naive Infected Patients: Cohort A

RXH Sticker	Sample Code eg A: 23	Extracted DNA QC	
		ug/ul	
		260/280 ratio	

Date:dd/mm/yy	Age:y/mm	Weight:kg	M
			F

Length: cm		
Clinical Stage: modified WHO		
CDC immunological class		
1	2	3

CD4 absolute x10^{9}/l	
CD4 %	
Viral Load c/ml	
Log Viral load	
Lactate mM/l	
Difficult Phlebotomy?	Y
	N

Current Oral Medication	
Drug	Duration

Current Major Event/Illness?	Yes	
	No	

Details

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Data Capture Form: HAART Treated Infected Patients: Cohort B

RXH Sticker	Sample Code eg B: 24	Extracted DNA QC	
		ug/ul	
		260/280 ratio	

Date:dd/mm/yy	Age:y/mm	Weight:kg	M
			F

Length: cm	
Clinical Stage: modified WHO	

CDC Immunological Class		
1	2	3

CD4 absolute x10⁹/l	
CD4 %	
Viral Load c/ml	
Log Viral load	
Lactate mM/l	
Difficult Phlebotomy?	Y
	N

Current Oral Medication - Include ARV's	
Drug	Duration

Have ARV's been changed since inception of HAART?	
Yes	
No	
Why?	
How and when?	

Drug compliance adequate?		
yes		
no		
based on	Adherence record	
	Viral load	

Current Major Event/Illness?	Yes	
	No	

Details/Comments

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Data Capture Form: Non-Infected Patients: Cohort C

RXH Sticker	Sample Code eg C: 24	Extracted DNA QC	
		ug/ul	
		260/280 ratio	

Date:dd/mm/yy	Age:y/mm	Weight:kg	M
			F

Length: cm

Current Oral Medication	
Drug	Duration

Details of procedure:

Current Event/Illness?	Yes	
	No	

Details

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Informed Consent Forms for the Study:

Mitochondrial:Nuclear DNA Ratios in Peripheral Blood Leukocytes and Lactate in Human Immunodeficiency-virus Infected Children on Antiretroviral Drug Regimens

University Of Cape Town

September 2004

Mitochondrial:Nuclear DNA Ratios in Peripheral Blood Leukocytes and Lactate in Human Immunodeficiency-virus Infected Children on Antiretroviral Drug Regimens

Investigators: George F van der Watt*, Howard Henderson*, Brian Eley †.
*Division of Chemical Pathology, Groote Schuur Hospital, University of Cape Town.
† Divisions of Paediatric Medicine and Infectious Diseases, Red Cross Childrens' Hospital, University of Cape Town

Information for Participating Patients
This study is being conducted to investigate specific side effects, due to Antiretroviral Drug treatment for HIV infection in children. Drug treatment may affect energy metabolism in certain patients by interfering with mitochondrial function. In the study we will measure the amount of mitochondrial DNA in white blood cells in healthy children and in HIV infected children to better understand these side effects. By learning more, we hope to be able to provide safer treatment for children with HIV infection.
Should you have any questions, you are free to contact Dr George van der Watt at Groote Schuur Hospital: Tel 021 404435 or Dr Brian Eley at Red Cross Hospital: Tel 021 6585111

Consent for DNA Analysis, Storage and Lactate Measurement: **Drug Treatment Patient Consent**

1. I ______________________________________ **(legal parent or guardian)** hereby give permission that blood may be taken and genetic material and plasma analysed, to try and determine the level of side effects due to antiretroviral drugs that may occur in ____ **(patient)** who will be receiving this medication as treatment for HIV infection

2. I understand that the genetic material will be obtained from blood (mark if applicable)

3. I request that no portion of the sample be stored for later use (mark if applicable)
OR
I request that a portion of the sample be stored indefinitely for:

- possible re-analysis
- research purposes, subject to the approval of the University of Cape Town Research Ethics Committee and that any information obtained will remain confidential

4. I request that the results of the analysis carried out on this sample of stored biological material will be made known to me via my doctor, in accordance with the relevant protocol

5. I have been informed that:

- the analysis is specific for measuring the amount of DNA and cannot determine the complete genetic makeup of an individual
- the investigators are under obligation to respect medical confidentiality
- where biological material is used for research purposes there may be no benefit to me or to my child

6. I UNDERSTAND THAT I MAY WITHDRAW MY CONSENT FOR ANY ASPECT OF THE ABOVE AT ANY TIME WITHOUT THIS AFFECTING MY OR MY CHILDS FUTURE MEDICAL CARE

7. ALL THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED

Parent or legal Guardian: Name __________________________ Date: ________/____/____
Sign __________________________ yyyy mm dd

Informed Consent obtained by: Name __________________________
Sign __________________________

- **This consent form is to be translated into Afrikaans and Xhosa**
- **A councillor is to explain and obtain each informed consent**
- **This form is to be retained by the Parent/Guardian of the Patient. A duplicate copy is to be kept in the patients folder**

University of Cape Town

Mitochondrial:Nuclear DNA Ratios in Peripheral Blood Leukocytes and Lactate in Human Immunodeficiency-virus Infected Children on Antiretroviral Drug Regimens

Investigators: George F van der Watt*, Howard Henderson*, Brian Eley †.
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Should you have any questions, you are free to contact Dr George van der Watt at Groote Schuur Hospital: Tel 021 404435 or Dr Brian Eley at Red Cross Hospital: Tel 021 6585111

Consent for DNA Analysis, Storage and Lactate Measurement: **ARV Naïve Patient Consent**

1. I ____________________________________ **(legal parent or guardian)** hereby give permission that blood may be taken and genetic material and plasma analysed, to measure the effect of HIV infection on mitochondrial DNA that may occur in _______________________________________ **(patient)**

2. I understand that the genetic material will be obtained from blood (mark if applicable)

3. I request that no portion of the sample be stored for later use (mark if applicable)

OR

I request that a portion of the sample be stored indefinitely for:

- possible re-analysis
- research purposes, subject to the approval of the University of Cape Town Research Ethics Committee and that any information obtained will remain confidential

4. I request that the results of the analysis carried out on this sample of stored biological material will be made known to me via my doctor, in accordance with the relevant protocol

5. I have been informed that:

- the analysis is specific for measuring the amount of DNA and cannot determine the complete genetic makeup of an individual
- the investigators are under obligation to respect medical confidentiality
- where biological material is used for research purposes there may be no benefit to me or to my child

6. I UNDERSTAND THAT I MAY WITHDRAW MY CONSENT FOR ANY ASPECT OF THE ABOVE AT ANY TIME WITHOUT THIS AFFECTING MY OR MY CHILDS FUTURE MEDICAL CARE

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Parent or legal Guardian: Name _________________________ Date: ________/____/____

Sign _________________________ yyyy mm dd

Informed Consent obtained by: Name _________________________

Sign _________________________

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Should you have any questions, you are free to contact Dr George van der Watt at Groote Schuur Hospital: Tel 021 404435 or Dr Brian Eley at Red Cross Hospital: Tel 021 6585111

Consent for DNA Analysis and Storage**: Control Group Consent**

1. I ____________________________________ **(legal parent or guardian)** hereby give permission that blood may be taken and genetic material analysed, for determination of the normal level of mitochondrial DNA in
_______________________________________ **(patient)**

2. I understand that the information obtained will be compared to that from HIV infected children to determine the effect of HIV and its treatment on mitochondrial DNA in these children (mark if applicable)

3. I understand that the genetic material will only be obtained from blood taken during routine management during his/her stay at Red Cross Childrens' Hospital (mark if applicable)

4. I request that no portion of the sample be stored for later use (mark if applicable)
OR
I request that a portion of the sample be stored indefinitely for:
- possible re-analysis
- research purposes, subject to the approval of the University of Cape Town Research Ethics Committee and that any information obtained will remain confidential

5. I request that the results of the analysis carried out on this sample of stored biological material will be made known an explained to me at my request, in accordance with the relevant protocol

6. I have been informed that:
- the analysis is specific for measuring the amount of DNA and cannot determine the complete genetic makeup of an individual
- the investigators are under obligation to respect medical confidentiality
- where biological material is used for research purposes there may be no benefit to me or to my child

7. I UNDERSTAND THAT I MAY WITHDRAW MY CONSENT FOR ANY ASPECT OF THE ABOVE AT ANY TIME WITHOUT THIS AFFECTING MY OR MY CHILDS FUTURE MEDICAL CARE

8. ALL THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED

Parent or legal Guardian: Name _________________________ Date: ____/____/_________
Sign _________________________ dd mm yyyy

Informed Consent obtained by: Name _________________________
Sign _________________________

- **This consent form is to be translated into Afrikaans and Xhosa**
- **A councillor is to explain and obtain each informed consent**
- **This form is to be retained by the Parent/Guardian of the Patient. A duplicate copy is to be kept in the patients folder**

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Ethics Approval Letter

University Of Cape Town

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
E53 Room 44.1, Old Main Building
Groote Schuur Hospital, Observatory,
7925
Queries : Xolile Fula
Tel : (021) 406-6492 Fax: 406-6411
E-mail : Xfula@curie.uct.ac.za

06 October 2004

REC REF: 124/2004

Dr GF Van Der Watt
Chemical Pathology

Dear Dr Van Der Watt

MITOCHONDRIAL-NUCLEAR DNA RATIOS IN PERIPHERAL BLOOD LEUKOCYTES AND LACTATE IN HUMAN IMMUNODEFICIENCY-VIRUS INFECTED CHILDREN ON ANTIRETROVIRAL DRUG REGIMENS

Thank you for your letter to the Research Ethics Committee dated 27 September 2004.

It is a pleasure to inform you that the Ethics Committee has approved the amended study protocol and three separate consent forms for the above study.

Please quote the REC. REF in all your correspondence

Yours sincerely

PROF. T. ZABOW
CHAIRPERSON

University of Cape Town

Technical Appendix

Development of the RT-PCR method for determination of mt:nDNA ratios in whole blood.

1. DNA extraction

DNA was extracted from 200 – 300µl of whole EDTA blood and purified by means of a Roche High Pure DNA extraction kit within 12 hours of phlebotomy. Extracted DNA was stored at -70°C until all collections were completed.

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2. Design of primers and hybridization probes

For each DNA extract, the nuclear gene for the human polymerase gamma accessory subunit (ASPOLG) – genebank accession number AF 142992, and the mitochondrial gene, human cytochrome-*c* oxidase subunit I (COX 1) – genebank accession number NC 001807 (mt complete genome region 5905 – 7446) were analysed on the NCBI gene database http://www.ncbi.nlm.nih.gov and the reported primers, and oligonucleotide probes checked to exclude errors before ordering the probes and primers. Oligo ™ software was used to check the position of primers, size the PCR product, position of oligonucleotide probes, exclude palindromic sequences within primers, melting point of primers, likelihood of primer-dimer formation and to ensure that the 3' end of each primer did not end on a 3rd wobble position with likely polymorphisms.

Human polymerase gamma accessory subunit (ASPOLG) sequence with highlighted regions delineated below

5'TGGTGGCTTGTTGGGATCCGTTGAGTGATGGGAGAGTGTGCTCTTTAACTTCGGAGAGAGATGCGCTCT
CGTGTAGCCGTCAGGGCCTGCCATAAGGTCTGCAGGTGCCTGTTGTCTGGGTTTGGGGGTCGAGTAG**ATG**C
GGGGCAGCCG**GAGCTGTTGACGGAAAGGAG**TAGCCCCAAAGGAGGGCATGTGAAGTCGCACGCGGAGCTC
GAGGGGAACGGCGAGCACCCAGAAGCCCCCGGGTCTGGAGAGGGAAGCGAGGCGCTGTTAGAGATCTGTC
AGAGAA**GGCATTTCCTAAGTGGAAGCAAGCA**GCAG**CTTAGCCGGGATTCTCTTCTG**AGTGGGTGCCACCC
CGGCTTCGGACCCTTGGGCGTAGAGTTGCGGAAGAACCTGGCCGCAGAATGGTGGACCTCGGTGGTGGTG
TTCAGGGAGCAGGTATTCCCGGTGGACGCCCTCCACCACAAACCAGGCCCTTTGCTACCCGGGGACAGTG
CCTTCAGGTTAGTTTCTGCAGAAACTCTACGCGAAATCTTGCAAGACAAAGAGCTGAGTAAGGAACAGCT
-3′

Start codon
Forward Primer
Reverse Primer
Flourescein labeled oligo
LC-Red 640 labeled oligo

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Human mitochondrial gene, cytochrome-*c* oxidase subunit I (COXI) sequence with highlighted regions delineated below

ATGTTCGCCGACCGTTGACTATTCTCTACAAACCACAAAGACATTGGAACACTATACCTATTATTCGGCG
CATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCGAGCCGAGCTGG**GCCAGCCAGGCAACCT**
TCTAGGT**AACGACCACATCTACAACGTTATCGTCAC**A**GCCCATGCATTTGTAATAATCTT**CTTCATAGTA
ATACCCATCATAATCGGAGGCTTTGGCAACTGACTAGTTCCCCTAATAATCGGTGCCCCCGATATGGCGT
TTCCCCGCATAAACAACATAAGCTTCTGACTCTTACCTCCCTCTCTCCTACTCCTGCTCGCATCTGCTAT

Start codon
Forward Primer
Reverse Primer
Flourescein labeled oligo
LC-Red 640 labeled oligo

3. PCR reactions and Light Cycler Settings

Real-time PCR was performed on a Roche Light-Cycler. A number of PCR reactions were assessed to ascertain the optimum PCR reaction temperatures and reagent concentrations to yield optimum standard and melting curves during the RT –PCR reaction for each gene. Final reagent mix is listed as follows:

<u>ASPOLG-2 and CCO1</u>

- 1µM Forward Primer
- 1µM Reverse Primer

- 0.4µM 5' LC-Red oligonucleotide
- 0.2µM 3' Fluorescein oligonucleotide
- 4µL Master Mix, Roche™ (consisting of Taq polymerase, bufferm MgCl, dNTP's containing dATP instead of dTTP)
- 5 µL sample DNA
- dH_2O to a final volume of 20µL

Final PCR conditions used were.

- 1 activation cycle: 90°C for 10 minutes
- 45 PCR cycles: denature: 95°C for 0 seconds; anneal: 60°C for 10 seconds with a single acquisition; extension: 72°C for 5 seconds
- Melting curves were used to evaluate PCR efficacy and continuity between runs as follows: denature: 95°C for 0 seconds; anneal: 55°C for 30 seconds followed by continuous acquisition of signal during a melt up to 80°C at a slope of 0.1°C/second
- Cooling cycle to reach 40°C for 30 seconds before termination

Data Analysis

Data was analysed using the second derivative maximum without baseline adjustment. Fluorescence was detected at F2/Back F1 to reduce background noise

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4. Standard Curves, quantitation of data and determination of the mitochondrial nuclear DNA ratio

Once the PCR reactions had been optimized, a control human DNA sample was prepared as follows:

- Five µL aliquots of extracted DNA from 27 healthy controls were pooled and the number of nuclear genome equivalents (NGEs) determined in the control mix
- A standard curve for the nuclear gene ASPOLG-2 was set up usingLight Cycler Control Kit DNA known to contain 15ng/µL of human DNA. Assuming that 1NGE = 3 pg of DNA this solution was calculated to contain 5000 NGEs /µL. This solution was then used to set up a standard curve for ASPOLG-2 ranging from 25 to 25 000 NGEs per PCR reaction
- This ASPOLG-2 standard curve thus generated was then used to determine the NGE value in the pooled control mix – The pooled control mix was thus calculated to contain 40 000 NGE's/5µL

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The control pooled human DNA thus obtained containing 40 000 NGEs/5µL was then used to set up standard curves for ASPOLG-2 in the range of 40 to 40 000 NGE's/5µL DNA per PCR reaction as demonstrated in Figure 1.

Because the amount of mtDNA in the pooled control was unknown it was arbitrarily assigned a value equivalent to the NGE value and so, the same control DNA was used to set up a standard curve for the mitochondrial COX1 gene using the same numerical values.

The mitochondrial/nuclear DNA ratio of the control mix was therefore arbitrarily set at 1 and every subsequent sample tested compared to this arbitrary ratio.

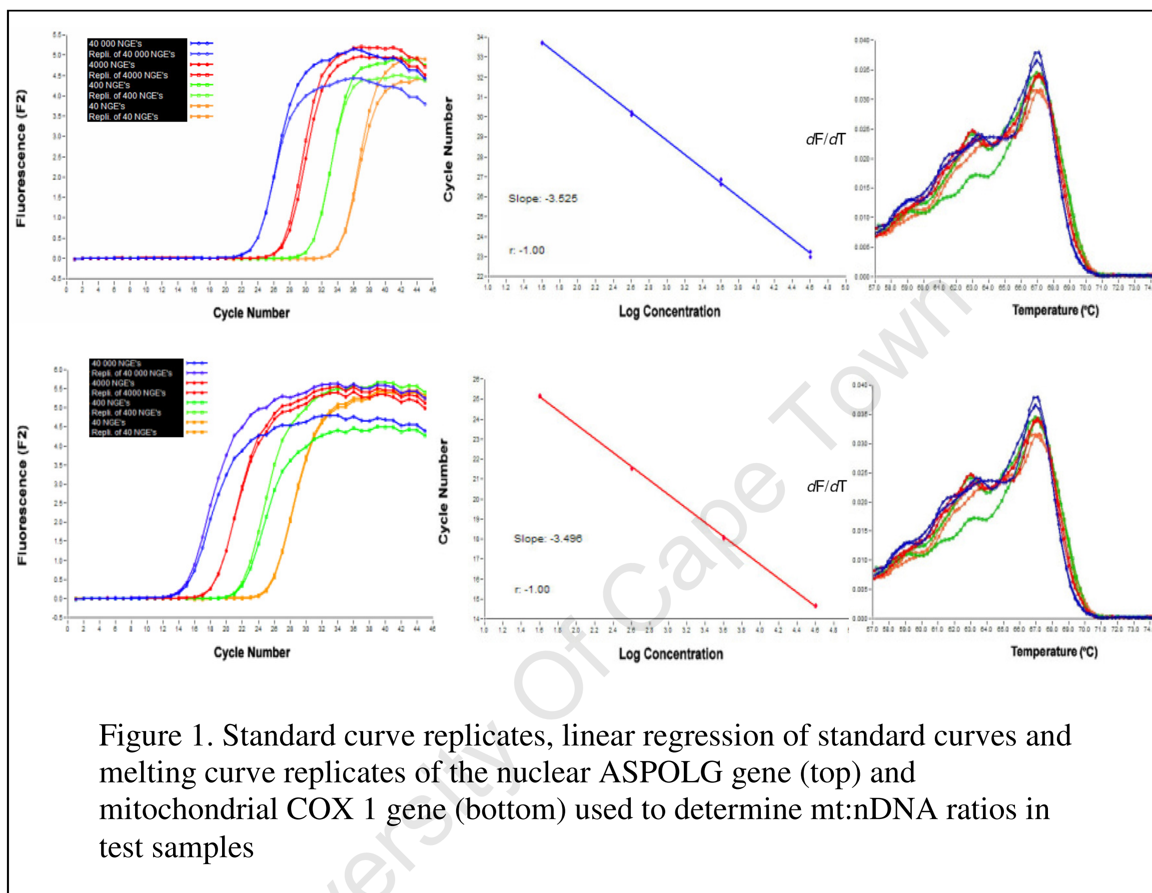

Figure 1. Standard curve replicates, linear regression of standard curves and melting curve replicates of the nuclear ASPOLG gene (top) and mitochondrial COX 1 gene (bottom) used to determine mt:nDNA ratios in test samples

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5. Determination of test sample mt:nDNA ratios

All RT-PCR reactions were performed in duplicate and the mean of two runs used. For each run on each day a new standard curve for each gene using 4 standards was generated in duplicate and test samples read off the standard curves.

The mean value for the mitochondrial gene divided by the mean value for the nuclear gene gave the calculated mt:nDNA ratio in each case.

A low and a high control taken from patient samples was included in each run and used to calculate the inter assay CV for each gene, a total of 19 separate runs were performed over 5 days in the study. Inter assay CVs were 5.1% for the mitochondrial gene, 7.4% for the nuclear gene and 8.5% for the mt:nDNA ratio, respectively.

Intra-assay CV was not calculated.

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Study Subject Raw Data

The following tables contain pertinent patient raw data divided into the following cohorts

Cohort A: HIV-infected patients, naïve to antiretroviral treatment

Cohort B: HIV-infected patients treated with antiretrovirals

Cohort C: Presumed HIV negative controls

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Cohort A: HIV-infected patients, naïve to antiretroviral treatment: 1

Cohort A: ARV Naïve

Code	Hospital Number	Sex	Weight(k	Height(c	Age (Mo)	Z height	Z weight	Z w/h	Date phleb	Difficult Phleb	Lactate	WCC abs X	CD4 % WCC	CD4 abs	CD4%Lymphs	Viral Load	WHO (
A1	88924055	m	9.75	75	26.48	-3.8	-2.49	-0.16	22/10/2004	n	2.5	14.7	7.56	1111	15.25	1 200 000	3
A2	13252390	m	3.25	57.7	3.94	-2.18	-3.43	-2.94	29/10/2004	n	1.3	12.21	7.70	940	21		2
A3	82304858	f	31.5	122.4	109	-1.58	0.39	3.04	29/10/2004	n	1.8	7.84	5.20	408	23.8		1
A4	89617039	f	12.2	85.2	44.02	-3.55	-1.95	0.24	2/11/2004	n	2.9	12.08	8.90	1075	18.6		2
A5	13193784	f	2.9	55.8	5.88	-3.76	-4.9	-3.04	2/11/2004	n	1.7	4.54	0.51	23	3.8	> 3 000 000	3
A6	85952299	f	17.7	112.6	77.4	-0.92	-1.14	-0.83	2/11/2004	y	3	7.98	3.50	279	6.1	150 000	2
A7	88186341	m	10.8	82.5	37.52	-3.49	-2.49	-0.72	2/11/2004	y	2	6.97	8.11	565	17.8		2
A8	83409938	f	18	112	94.25	-2.27	-1.89	-0.53	2/11/2004	n	2	7.82	10.81	845	22.3		2
A9	13083514	f	7.6	71	12.55	-1.39	-1.96	-1.29	2/11/2004	n	1.8	8.17	11.51	940	15.7	5 300	2
A10	12994240	m	7.9	73	20.83	-3.72	-3.27	-1.84	2/11/2004	n	1.8	16.48	3.80	626	7.3	300 000	3
A11	13150701	f	14	93.8	52.33	-2.48	-1.45	0.12	2/11/2004	n	1.8	9.61	9.40	903	21.4		1
A12	12483038	m	7.5	69	12.22	-2.72	-2.66	-1.03	5/11/2004	n	2.4	8.19	5.96	488	10.3		2
A13	85978914	m	20.18	104	72.86	-2.57	-0.28	2.1	5/11/2004	n	2	6.24	6.83	426	17.4		2
A14	87346623	m	14.5	94	42.94	-1.4	-0.75	0.19	9/11/2004	n	2.2	9.6	7.27	698	6.68	510 000	2
A15	13369517	m	5.8	66.5	3.38	1.64	-0.47	-2.43	9/11/2004	n	2.8	14.61	5.98	874	15.6		2
A16	89747885	m	12.4	85.2	36.99	-2.71	-1.48	0.16	9/11/2004	n	2.3	6.62	27.21	1801	45.8		1
A17	88800628	f	11.2	86.2	36.99	-2.24	-2.05	-0.82	9/11/2004	n	2.3	8.07	8.62	696	16.4		1
A18	12596656	m	7.94	71	10.84	-1.39	-1.89	-1.15	9/11/2004	n	2.2	8.95	1.90	170	11.4	1 600 000	3
A19	86818739	f	22.4	105	102.4	-1.51	-1.1	0.22	9/11/2004	n	2.1	9.35	23.30	2179	39.5		1
A20	88772140	m	10.2	78.3	23.19	-2.6	-1.71	-0.42	9/11/2004	n	2.6	5.16	12.09	624	19.4	470 000	3
A21	85586394	m	20.4	119	88.99	-0.95	-1.22	-0.78	09/11/2004	n	2.1	21.69	1.68	364	22.2		1
A22	88265798	f	18.2	114.7	89.65	-1.5	-1.62	-0.93	16/11/2004	n	1.1	7.05	3.66	258	12.4		1
A23	87277786	f	16.8	103	69.35	-2.14	-1.05	0.36	16/11/2004	y	3.7	4.73	1.86	88	3.41	18 000	2
A24	86061967	m	15	102	55.98	-1.27	-1.47	-0.91	16/11/2004	y	2.8	7.76	10.70	830	20.9		2
A25	83215590	f	19.4	119.3	119.15	-2.72	-2.45	-1.16	30/11/2004	n	1.3	7.13	0.87	62	5.68		2
A26	89054118	m	7.52	77.5	20.73	-2.3	-3.56	-3.45	30/11/2004	n	1.8	6.11	2.14	131	6.08		3
A27	89061360	m	10.4	82	24.54	-1.26	-1.78	-1	30/11/2004	n	2.2	9.11	7.01	639	14.5	260 000	3
A28	13340336	f	5.56	60	5.45	-1.89	-1.58	0.03	30/11/2004	n	2.6	16.99	11.10	1886	17.1	1 400 000	3
A29	88854849	m	11.4	89.1	45.8	-2.98	-2.66	-1.44	30/11/2004	n	2.5	10.34	4.32	447	14		2
A30	12564621	f	6.6	75.5	11.79	0.51	-2.73	-3.6	3/12/2004	n	2.3	11.13	12.70	1413	30.5		1
A31	88961958	f	16	106.7	72.44	-1.65	-1.59	-0.81	18/1/2005	y	2.3	8.43	6.74	568	9.91		2
A32	84536465	m	22.6	118.6	82.42	-0.47	0.01	0.41	18/1/2005	n	2.8	6.49	8.95	581	16.6	33 000	2
A33	13099726	m	6.36	65.5	8.48	-2.32	-2.71	-1.26	18/1/2005	n	1.9	6.74	3.19	215	4.63	650 000	3
A35	13088992	f	7.02	71.1	11.53	-0.93	-2.3	-2.03	18/1/2005	n	2	4.1	8.12	333	18.5		2
A36	88065750	f	8.25	77.4	35.18	-4.32	-3.93	-1.95	18/1/2005	y	2.8	17.7	3.78	669	7.4	280 000	3
A37	87338208	m	13.6	85.6	49.38	-4.23	-1.73	0.93	25/1/2005	n	1.6	8.07	18.80	1517	30.7		2
A38	82404401	f	20	122	109.03	-1.64	-1.98	-1.37	25/1/2005	n	1.3	9.86	3.11	307	7.72		2
A39	82483892	f	20.8	124	109.03	-1.34	-1.8	-1.37	25/1/2005	n	1.5	7.4	3.69	273	8.74		1
A40	88696638	f	10.5	80	28.29	-2.37	-1.69	-0.28	25/1/2005	y	3.3	15.85	8.92	1414	16.9		2
A41	13198429	m	24.8	123.5	82.62	0.48	0.62	0.42	25/1/2005	n	1.6	6.93	6.81	472	15.7		3

Cohort A: HIV-infected patients, naïve to antiretroviral treatment: 2

Cohort A: ARV Naïve

Code	Hospital Number	Sex	Weight(k	Height(c	Age (Mo)	Z height	Z weight	Z w/h	Date phleb	Difficult Phleb	Lactate	WCC abs X	CD4 % WCC	CD4 abs	CD4%Lymphs	Viral Load	WHO (
A42	13761499	f	5.66	70	22.4	-4.67	-4.88	-3.41	1/2/2005	n	2.1	3.848	24.06	926			2
A43	87047973	f	16.8	102.8	72.21	-2.42	-1.22	0.39	1/2/2005	y	2.6	3.079	13.32	410			2
A44	88977830	f	20.8	101.2	42.21	0.81	2.62	3.04	1/2/2005	n	1.3	3.228	23.73	766			2
A45	13020110	f	5.86	61.8	8.08	-2.73	-2.49	-0.29	1/2/2005	n	2.1	4.81	15.95	767		1 400 000	3
A46	13029798	m	12.6	84	21.81	-0.56	0.32	0.85	1/2/2005	n	1.3	3.627	15.49	562			2
A47	89552517	f	9.32	79.8	26.41	-2.03	-2.4	-1.38	1/2/2005	y	2.4	7.547	14.99	1131		690 000	2
A48	89548762	f	9.6	75.5	17.28	-1.54	-0.94	-0.12	1/2/2005	n	1.5			936	19.45	660 000	2
A49	13444021	f	4.76	60.7	6.21	-2.11	-2.91	-1.56	1/2/2005	n	2.4	7.858	8.21	645			3
A50	11458031	m	10.25	70.2	14.68	-3.14	-0.51	1.96	1/2/2005	n	1.2	10.61	6.52	692	15.4		2
A51	89233423	f	11.4	86	27.79	-0.5	-0.93	-0.6	1/2/2005	n	1.2	14.19	8.12	1152	21.4		2
A52	89543813	m	9.84	78	18.82	-1.67	-1.49	-0.78	1/2/2005	n	0.8	17.61	5.41	953	7.65	3 000 000 +	2
A53	83215590	f	20	119	121.32	-2.92	-2.4	undefin	4/2/2005	n	1.5	7	0.89	62	5.68	96 000	2
A54	11434693	f	20.2	117	96.12	-1.56	-1.34	-0.24	4/2/2005	n	1.8	17.6	1.44	254	11	64 000	2
A55	86736717	m	13.4	96	51.74	-2.11	-1.99	-1.02	8/2/2005	y	2.4	7.32	12.50	915	20.9		2
A56	10006773	f	6.76	80	18.36	-0.38	-3.51	-4.37	8/2/2005	n	2.2	10.37	12.60	1307	27.12		2
A57	89426878	f	7.07	70	20.11	-4.12	-3.45	-1.64	11/2/2005	y	2.4	6.5	5.17	336	4.4	3 000 000	2
A58	12668224	f	5.54	69	9.99	-1	-3.39	-3.38	15/2/2005	n	2	7.11	4.66	331	8.54		3
A59	13398367	m	9.2	79.5	20.01	-1.98	-2.35	-1.88	15/2/2005	n	2.5	8.74	10.10	883	19.8		1
A60	81046419	f	22.8	124.7	120.4	-2.02	-1.85	undefin	15/2/2005	n	2.1	3.5	12.74	446	11.2	35000	2
A61	13659958	f	7.245	73	16.2	-2.03	-2.84	-2.27	4/3/2005	n	2.5	11.74	11.91	1398	17.6	424000	2
A62	13976139	m	15.5	105	82.79	-3.19	-2.75	-1.1	18/3/2005	y	2.8	7.71	0.83	64	2.78		2

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Cohort A: HIV-infected patients, naïve to antiretroviral treatment: 3

Cohort A: ARV Naïve

Code	COX-1 a1	COX1 a2	COX-1 b1	COX1 b2	COX-1 me	ASPOLG-2 a1	ASPOLG a2	ASPOLG-2 b1	ASPOLG b2	ASPOLG-2 mea	mt/nDNA Ratio
A1	393	393	428	428	411	1520	1520	1733	1733	1627	**0.252**
A2	1738	1784	2104	2160	1947	2991	2701	3011	2720	2856	**0.682**
A3	4178	3844	5060	4656	4435	7052	6370	7479	6756	6914	**0.641**
A4	764	735	926	890	829	1256	1134	1299	1173	1216	**0.682**
A5	1990	1946	1626	1591	1788	3038	3209	3015	3184	3112	**0.575**
A6	800	800	970	969	885	1680	1518	1538	1389	1531	**0.578**
A7	1227	1486	1286	1557	1389	2069	1869	2244	2027	2052	**0.677**
A8	1005	1042	1218	1262	1132	1980	1788	2255	2037	2015	**0.562**
A9	1835	2223	1815	2198	2018	2498	2256	2497	2255	2377	**0.849**
A10	4751	4648	5437	5319	5039	9598	10140	10100	10670	10127	**0.498**
A11	4813	4708	4536	4438	4624	4082	4311	3994	4218	4151	**1.114**
A12	1855	2247	1821	2205	2032	3410	3043	3368	3080	3225	**0.630**
A13	2913	3529	3029	3669	3285	6436	5940	6576	5814	6192	**0.531**
A14	3161	3828	3136	3798	3481	4826	4221	4673	4359	4520	**0.770**
A15	2811	3268	2698	3404	3045	6509	5465	6051	5879	5976	**0.510**
A16	3628	4183	3454	4394	3915	3416	3085	3599	3251	3338	**1.173**
A17	2030	2154	2247	2385	2204	2409	2120	2409	2120	2265	**0.973**
A18	2081	2113	2304	2339	2209	1687	1636	x	x	1662	**1.330**
A19	2764	2729	3061	3021	2894	3820	4267	x	x	4044	**0.716**
A20	1892	1976	2095	2188	2038	2039	2199	x	x	2119	**0.962**
A21	2295	2398	2542	2655	2473	5700	5578	x	x	5639	**0.438**
A22	924	958	1023	1061	992	1939	1717	x	x	1828	**0.542**
A23	1058	1029	1172	1139	1100	902	863	x	x	883	**1.246**
A24	4031	3943	4386	4290	4163	5612	5927	5360	5661	5640	**0.738**
A25	721	691	799	766	744	949	982	x	x	966	**0.771**
A26	1071	1041	1186	1153	1113	1468	1379	x	x	1424	**0.782**
A27	1798	1811	1991	2005	1901	1665	1507	x	x	1586	**1.199**
A28	2884	2717	3194	3008	2951	3901	3651	x	x	3776	**0.781**
A29	4799	4695	4655	4554	4676	4075	4303	3996	4220	4149	**1.127**
A30	1881	1886	2083	2089	1985	2426	2426	x	x	2426	**0.818**
A31	581	575	644	637	609	783	927	x	x	855	**0.713**
A32	841	828	931	916	879	1044	1056	x	x	1050	**0.837**
A33	2013	1993	1984	1964	1989	1789	1668	1874	1748	1770	**1.124**
A35	317	313	327	308	316	268	261	273	280	271	**1.169**
A36	4103	3926	3994	3858	3970	4613	4872	4446	4696	4657	**0.853**
A37	1101	948	1085	935	1017	1375	1191	1440	1247	1313	**0.775**
A38	687	677	677	667	677	1253	1068	1119	1313	1188	**0.570**
A39	1138	1114	1121	1098	1118	1356	1315	1420	1378	1367	**0.818**
A40	1550	1542	1528	1520	1535	2140	2168	2242	2271	2205	**0.696**
A41	1873	1832	1595	1560	1715	2335	2466	2104	2222	2282	**0.752**

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Cohort A: HIV-infected patients, naïve to antiretroviral treatment: 4

Cohort A: ARV Naïve

Code	COX-1 a1	COX1 a2	COX-1 b1	COX1 b2	COX-1 me	ASPOLG-2 a1	ASPOLG a2	ASPOLG-2 b1	ASPOLG b2	ASPOLG-2 mea	mt/nDNA Ratio
A42	2041	1820	2011	1793	1916	1653	1585	1731	1660	1657	**1.156**
A43	564	565	556	557	561	464	407	486	419	444	**1.262**
A44	929.4	909	860.7	842	885	786	830	767.3	810	798	**1.109**
A45	1175	1175	1281	1281	1228	750	750	850	850	800	**1.535**
A46	416	445	409	438	437	391	414	410	458	418	**1.045**
A47	1774	1573	1749	1551	1662	1153	1174	1208	1230	1191	**1.395**
A48	857	749	844	738	797	704	737	736	772	737	**1.081**
A49	753	797	785	742	769	945	1031	863	990	1080	**0.712**
A50	493	478	485	471	482	610	738	557	773	639	**0.754**
A51	1461	1502	1547	1590	1525	2468	2166	2340	2667	2410	**0.633**
A52	2860	2583	3027	2734	2801	1802	1768	1947	1910	1857	**1.509**
A53	957.2	936	1083	1059	1009	716	756	675.5	713	715	**1.411**
A54	1179	1170	1248	1238	1209	801	646	865	698	753	**1.606**
A55	305.7	299	300.3	294	300	626	661	589.7	623	625	**0.480**
A56	307	322	325	341	324	514	514	555	555	535	**0.606**
A57	1379	1364	1459	1444	1412	1510	1494	1632	1615	1563	**0.903**
A58	1656	1652	1752	1749	1702	1562	1457	1687	1574	1570	**1.084**
A59	2574	2425	2566	2725	2573	3742	3503	4043	3786	3769	**0.683**
A60	1011	1027	1069	1087	1049	784	765	848	827	806	**1.301**
A61	2607	2395	2759	2535	2574	2577	2618	2784	2829	2702	**0.953**
A62	1138	1079	1205	1142	1141	1613	1357	1743	1466	1545	**0.739**

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Cohort B: HIV-infected patients treated with antiretrovirals: 1

Cohort B: ARV Exposed

Code	Number	Sex	Weight(k	Length(c	Age (	Age (y	Z-Height	Z-Weight	W/H	Onset of RX	Duration c	AZT	3TC	D4T	RIT	EFV	Nevirapine	Kaletra	Date phleb	Difficult Phleb	Lactate
B1	89460349	m	10.96	crf	13.8	1.15		0.32		5/1/2004	9.56	AZT	3TC		RIT				22/10/2004	n	1.6
B2	89408280	m	15.6	crf	22.1	1.84		2.64		18/2/2004	8.11		3TC	D4T	RIT				22/10/2004	n	1.4
B3	86220647	m	15.5	97.1	61.4	5.12	-2.69	-1.21	0.56	11/3/2004	7.39		3TC	D4T		EFV			22/10/2004	n	1.4
B6	10030575	m	11.7	84.7	10.2	0.85	4.1	1.98	-0.89	26/2/2004	7.85		3TC	D4T	RIT				22/10/2004	n	1.7
B8	88847843	f	10.6	80	29.7	2.47	-2.64	-1.77	-0.18	25/7/2003	15.18			D4T		EFV			29/10/2004	n	2.3
B10	88985759	f	9.57	75.5	19.1	1.59	-2.1	-1.23	-0.15	25/2/2004	8.11	AZT	3TC		RIT				29/10/2004	y	3
B13	89633853	m	10.46	74	14.4	1.2	-1.7	-0.26	0.99	25/11/2003	11.14	AZT	3TC		RIT				29/10/2004	n	1.9
B15	86728359	m	18.5	101	49	4.08	-0.6	0.79	1.62	17/10/2003	12.55		3TC	D4T		EFV			2/11/2004	y	1.6
B16	85691624	f	16	106	69.9	5.83	-1.57	-1.45	-0.68	4/7/2003	16		3TC	D4T		EFV			2/11/2004	n	1.7
B17	81116543	m	27	130	130	10.8	-1.65	-1.26	0.07	2/6/2003	17.05		3TC	D4T		EFV			2/11/2004	n	1.6
B18	80254527	m	22.2	122.1	125	10.4	-2.82	-2.15	-0.45	22/7/2003	15.41	AZT	3TC					Kaletra	2/11/2004	n	2
B20	89355861	f	8.96	74.2	18.1	1.51	-2.21	-1.6	-0.54	19/8/2003	14.49	AZT	3TC		RIT				2/11/2004	n	1.7
B21	88821756	m	7.8	71.4	23.3	1.94	-4.7	-3.54	-1.47	30/6/2003	16.23		3TC	D4T	RIT				5/11/2004	n	2.1
B22	87665931	f	16.7	92	43.7	3.64	-1.78	0.62	2.25	3/6/2003	17.12		3TC	D4T		EFV			5/11/2004	y	3.2
B23	89379721	f	8.7	77.9	18.7	1.56	-1.2	-1.91	-1.72	21/11/2003	11.5	AZT	3TC		RIT				5/11/2004	n	2.3
B24	87012910	f	14.4	100	61.6	5.14	-2.07	-1.78	-0.71	5/11/2002	24.01		3TC	D4T	RIT				5/11/2004	n	2
B25	86983715	f	18.6	109.4	72	6	-1.07	-0.41	0.28	5/11/2002	24.15		3TC	D4T		EFV			9/11/2004	n	1.4
B26	85537801	m	17.6	104	63.7	5.31	-1.69	-0.77	0.47	29/5/2003	17.41		3TC	D4T		EFV			9/11/2004	n	2.4
B27	85997955	f	21.6	110	74.7	6.23	-1.19	0.46	1.72	27/5/2003	17.71		3TC	D4T		EFV			16/11/2004	n	2.3
B28	86110459	m	16.6	100	72.7	6.06	-3.37	-1.79	0.56	3/6/2003	17.48		3TC	D4T		EFV			16/11/2004	n	1.8
B29	89511406	m	12.68	73	20.1	1.67	-3.57	0.65	3.8	19/5/2004	6.04		3TC	D4T	RIT				19/11/2004	y	2.3
B30	89087035	m	7.04	75	20.3	1.7	-2.99	-3.92	-3.47	19/11/2003	12.02		3TC	D4T	RIT				19/11/2004	n	2.3
B31	87970190	m	13.7	90	40.8	3.4	-2.06	-1.02	0.3	24/03/2003	19.91		3TC	D4T	RIT				19/11/2004	n	2
B32	89543094	f	9.33	76.7	16	1.33	-0.71	-0.98	-0.72	24/11/2003	11.86		3TC	D4T	RIT				19/11/2004	n	2.4
B33	86699485	m	14.7	94	65.3	5.44	-3.95	-2.2	0.31	19/11/2002	24.01		3TC	D4T		EFV			19/11/2004	y	1.9
B34	88207584	m	21.6	116	102	8.48	-2.1	-1.27	0.59	11/2/2003	21.48		3TC	D4T		EFV			26/11/2004	y	2.7
B35	12579157	f	9	74	19.3	1.61	-2.64	-1.74	-0.44	22/6/2004	5.39		3TC	D4T	RIT				3/12/2004	y	3.1
B36	89285951	m	10.6	80	21.3	1.78	-1.66	-1.18	-0.36	3/12/2003	12.02		3TC	D4T	RIT				3/12/2004	y	2.3
B37	88556394	f	29.2	122	98.7	8.22	-0.93	0.63	2.34	3/6/2004	6.01		3TC	D4T		EFV			3/12/2004	n	1.5
B38	11517919	f	10.2	75	21.1	1.76	-2.78	-0.98	0.75	3/5/2004	7.03	AZT	3TC		RIT				3/12/2004	n	2.4
B39	87220760	m	13	82.8	53.6	4.47	-5.32	-2.3	0.95	3/12/2003	12.02	AZT	3TC			EFV			3/12/2004	y	2.1
B40	89677488	m	12	76	15	1.25	-1.19	0.96	2.12	3/12/2003	12.02		3TC	D4T			Nevirapine		3/12/2004	y	2.5
B41	89580138	m	8.86	73	16	1.33	-2.56	-1.98	-0.61	20/05/2004	6.47	AZT	3TC		RIT				3/12/2004	y	1.9
B42	88597034	m	11.9	84.7	27.1	2.26	-1.02	-0.83	-0.14	3/6/2003	18.04		3TC	D4T	RIT				3/12/2004	n	1.3
B43	86818267	m	18	103.4	70.6	5.88	-2.49	-1.06	0.84	12/9/2003	16.23		3TC	D4T		EFV			18/1/2005	y	2.3
B44	12638250	f	7.6	69.3	11.5	0.96	-1.56	-1.73	-0.75	13/7/2004	6.21		3TC	D4T				Kaletra	18/1/2005	y	3.3
B45	86137155	m	19	102	58.4	4.87	-1.55	0.25	1.75	25/3/2003	21.85		3TC	D4T		EFV			18/1/2005	n	1.9
B46	89706519	f	9.2	72	13.8	1.15	-1.51	-0.71	0.39	18/7/2004	6.04		3TC	D4T	RIT				18/1/2005	n	2.6
B47	10011286	f	8.6	76	26.4	2.2	-3.15	-2.98	-1.32	9/7/2004	6.34		3TC	D4T				Kaletra	18/1/2005	n	1.7
B48	89202360	f	12.5	80	23	1.92	-1.73	0.57	2.13	16/7/2003	18.36		3TC	D4T	RIT				25/1/2005	y	2.3

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Cohort B: HIV-infected patients treated with antiretrovirals: 2

Cohort B: ARV Exposed

Code	Number	Sex	Weight(k	Length(c	Age (	Age (y	Z-Height	Z-Weight	W/H	Onset of RX	Duration c	AZT	3TC	D4T	RIT	EFV	Nevirapine	Kaletra	Date phleb	Difficult Phleb	Lactate
B49	85406015	m	17.4	105	74.2	6.18	-2.48	-1.54	0.16	2/12/2003	13.8		3TC	D4T		EFV			25/1/2005	y	2
B50	86959103	m	17	100	48.9	4.07	-0.81	0.08	0.82	25/03/2003	22.08		3TC	D4T		EFV			25/1/2005	n	1.6
B51	87619953	f	11.6	81	37.6	3.13	-3.72	-1.84	0.46	22/6/2004	7.13	AZT	3TC					Kaletra	25/1/2005	y	2.8
B52	89183107	f	18.1	93	22.4	1.86	2.46	5.24	4.76	26/7/2003	18.13		3TC	D4T			Nevirapine		28/1/2005	y	1.9
B53	89119663	f	6.52	67.5	21.6	1.8	-5.27	-4.09	-1.54	20/7/2004	6.31		3TC	D4T				Kaletra	28/1/2005	n	1.5
B54	12545539	m	10.85	75.2	15.8	1.32	-1.74	-0.21	1.03	27/7/2004	6.21	AZT	3TC					Kaletra	1/2/2005	n	1.9
B55	84988948	m	18	109.9	79.9	6.66	-1.98	-1.63	-0.39	19/2/2004	11.43		3TC	D4T		EFV			1/2/2005	y	2.6
B56	84265537	m	50.4	128	90.1	7.51	0.68	6.35	7.48	17/8/1998	77.63		3TC	D4T		EFV			4/2/2005	n	3.3
B57	89583108	m	9.8	76	16.5	1.37	-1.67	-1.21	-0.3	20/7/2004	6.54		3TC	D4T	RIT				4/2/2005	n	2.7
B58	87993622	m	13.9	91	34.1	2.84	-0.68	-0.25	0.28	10/2/2004	11.83		3TC	D4T		EFV			4/2/2005	n	1.8
B59	89583330	m	7.15	73.4	19.3	1.61	-3.28	-3.76	-2.91	27/7/2004	6.31		3TC	D4T	RIT				4/2/2005	n	2.7
B60	10038768	m	8.9	76	14.9	1.24	-1.15	-1.79	-1.41	10/2/2004	11.96	AZT	3TC		RIT				8/2/2005	n	2.2
B61	89173702	f	16.6	106.5	60.9	5.07	-0.53	-0.61	-0.38	6/7/2004	7.13		3TC	D4T		EFV			8/2/2005	n	2.1
B62	85985281	m	17.2	102	70.1	5.84	-2.73	-1.38	0.59	9/9/2003	17.02		3TC	D4T		EFV			8/2/2005	n	2.5
B63	85308591	f	26	124.5	100	8.35	-0.63	-0.01	0.69	8/8/2003	18.07		3TC	D4T		EFV			8/2/2005	n	1.7
B64	12674073	m	8.1	69	12.3	1.02	-2.73	-2.08	-0.23	20/8/2004	5.75		3TC	D4T	RIT				11/2/2005	n	2.1
B65	88279336	f	13.8	92	38.7	3.23	-1	-0.49	0.28	16/8/2002	29.89		3TC	D4T	RIT				11/2/2005	n	1.3
B66	89740328	m	5.74	67	15.2	1.27	-4.42	-4.72	-2.7	12/8/2004	6.01		3TC	D4T	RIT				11/2/2005	n	2.6
B67	87048690	m	18	96.5	48.1	4.01	-1.53	0.63	2.13	25/2/2003	23.69		3TC	D4T		EFV			15/2/2005	n	1.8
B68	87746467	f	18.2	101	45	3.75	0.3	1.18	1.53	9/9/2003	17.25		3TC	D4T		EFV			15/2/2005	n	1.9
B69	89262489	f	9.8	77	22.5	1.88	-2.51	-1.51	-0.27	15/8/2003	18.07		3TC	D4T	RIT				15/2/2005	n	2.2
B70	87761821	f	15	93	57.8	4.81	-3.46	-1.59	0.7	25/2/2003	24.01		3TC	D4T	RIT				25/2/2005	n	1.2
B71	85807774	m	16.8	100	69	5.75	-3.11	-1.53	0.69	13/7/2004	7.46		3TC	D4T		EFV			25/2/2005	n	1.4
B72	87502126	f	18.4	103	57	4.75	-87	-0.43	1.28	10/9/2004	5.52		3TC	D4T		EFV			25/2/2005	y	2.6
B73	86932472	f	17.2	90	47.3	3.94	-2.78	0.57	2.97	16/10/2001	40.57	AZT	3TC		RIT				4/3/2005	n	1.6
B74	11440757	m	8.2	74	19.4	1.62	-3.1	-2.9	-1.74	3/8/2004	7	AZT	3TC					Kaletra	4/3/2005	n	2.1
B75	87916193	m	15.5	94.5	50.3	4.19	-2.27	-0.81	0.79	25/2/2003	24.24		3TC	D4T		EFV			4/3/2005	n	2.4
B76	89633853	m	11.3	79.2	18.5	1.54	-1.21	-0.23	0.58	25/11/2003	15.28	AZT	3TC					Kaletra	4/3/2005	n	2.1
B77	88207303	f	14.8	89	36	3	-1.32	0.36	1.47	20/2/2004	12.42		3TC	D4T		EFV			4/3/2005	y	2.3
B78	89127526	m	15.1	89.5	33.4	2.79	-0.96	0.53	1.34	11/9/2003	17.97	AZT	3TC			EFV			11/3/2005	y	2
B79	11513744	m	13.4	81	19.3	1.61	-0.84	1.35	2,31	8/4/2004	11.3	AZT	3TC		RIT				18/3/2005	n	1.8
B80	89565584	f	10.2	76.3	27.6	2.3	-3.31	-1.84	0.14	1/10/2004	5.52	AZT	3TC		RIT				18/3/2005	n	1.9
B81	88028303	f	13.2	89.2	46	3.83	-2.81	-1.46	0.32	7/10/2003	17.35	AZT	3TC			EFV			18/3/2005	n	1.7
B82	**88924055**	m	13.6	90.3	32.9	2.74	-0.63	-0.3	0.18	22/10/2004	6.37	AZT	3TC			EFV			4/5/2005		
B83	**85952299**	f	19.8	115	82.9	6.91	-0.93	-0.67	-0.06	2/11/2004	6.1		3TC	D4T		EFV			19/4/2005		
B84	**13083514**	f	10.15	80	19.2	1.6	-0.68	-0.71	-0.46	2/11/2004	6.67	AZT	3TC					Kaletra	24/05/2005		
B85	**12994240**	m	9.67	75.6	27	2.25	-3.72	-2.59	-0.37	2/11/2004	6.21		3TC	D4T				Kaletra	10/5/2005		
B86	**87346623**	m	16.9	100	48.9	4.08	-0.82	0.03	0.76	9/11/2004	6		3TC	D4T		EFV			27/5/2005		
B87	**12596656**	m	9.78	76.2	16.6	1.38	-1.64	-1.25	-0.38	9/11/2004	5.9	AZT	3TC		RIT				03/05/2005		
B88	**88772140**	m	11.45	85	29.2	2.43	-1.38	-1.38	-0.57	9/11/2004	6.01		3TC	D4T	RIT				11/5/2005		

Cohort B: HIV-infected patients treated with antiretrovirals: 3

Cohort B: ARV Exposed

Code	Number	Sex	Weight(k	Length(c	Age (	Age (y	Z-Height	Z-Weight	W/H	Onset of RX	Duration c	AZT	3TC	D4T	RIT	EFV	Nevirapine	Kaletra	Date phleb	Difficult Phleb	Lactate
B89	**87277786**	f	18.4	103	76.6	6.38	-2.72	-0.8	1.28	16/11/2004	7.23		3TC	D4T		EFV			24/6/2005		
B90	**89061360**	m	12.8	88.5	30.2	2.51	-0.58	-0.54	-0.1	30/11/2004	5.9		3TC	D4T		EFV			20/05/2005		
B91	**13340336**	f	7.54	68	11	0.91	-1.80	-1.66	-0.38	30/11/2004	6		3TC	D4T	RIT				17/05/2005		
B92	**13099726**	m								18/1/2005	5.8		3TC	D4T	RIT				31/08/2005		
B93	**89543813**	m	12.05	84	24.6	2.05	-0.65	-0.32	0.14	1/2/2005	6.2		3TC	D4T		EFV			27/7/2005		
B94	**83215590**	f	19	121	127	10.6	-3.04	-2.74	undefin	4/2/2005	6.5		3TC	D4T		EFV			28/7/2005		
B95	**11434693**	f	20.6	118	101	8.41	-1.72	-1.48	-0.23	4/2/2005	6		3TC	D4T		EFV			29/06/2005		
B96	**89426878**	f								11/2/2005	5.9		3TC	D4T		EFV			19/08/2005		
B97	**89617039**	f								01/03/2005	6		3TC	D4T		EFV			30/08/2005		

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Cohort B: HIV-infected patients treated with antiretrovirals: 4

Cohort B: ARV Exposed

Code	WCC CD45	CD4% WCC	CD4 abs X	CD4% Lymphs	Viral Load F	log vl	COX-1 a	COX-1 b	COX-1 Mean	ASPOLG-2 a	ASPOLG-2 b	ASPOLG-2 Mean	mt:n Ratio
B1	4.27	15.69	670	25.4	61000	4.79	883	920	902	905	1102	1004	**0.90**
B2	6.84	9.44	646	23.7	250000	5.40	3406	3551	3479	4263	5187	4725	**0.74**
B3	7.51	8.30	623	24.9	24	1.38	107	111	109	215	262	239	**0.46**
B6	11.16	17.50	1953	33.6	1300	3.11	364	379	372	424	516	470	**0.79**
B8	6.35	17.40	1105	24.3	220000	5.34	895	934	915	1945	2367	2156	**0.42**
B10	13.99	6.90	965	11.4	42000	4.62	5713	5956	5835	5225	6358	5792	**1.01**
B13	7.46	14.71	1097	21.2	24	1.38	726	757	742	623	758	691	**1.07**
B15	6.8	11.66	793	23.26	24	1.38	550	574	562	567	690	629	**0.89**
B16	4.84	16.05	777	17.16	24	1.38	537	560	549	1034	1258	1146	**0.48**
B17	7.07	13.39	947	23.4	24	1.38	1024	1078	1051	1505	1831	1668	**0.63**
B18	8.69	3.90	339	11.2	7600	3.88	808	842	825	1454	1769	1612	**0.51**
B20	9.94	14.10	1402	31.7	320000	5.51	1506	1579	1543	1856	2258	2057	**0.75**
B21	17.12	2.83	484	9.85	2700	3.43	3777	3937	3857	7921	9638	8780	**0.44**
B22			2354	34.12	24	1.38	5911	5843	5877	7730	7210	7470	**0.79**
B23	8.42	16.20	1364	25.5	24	1.38	2260	2356	2308	2523	3070	2797	**0.83**
B24	12.62	10.70	1350	14.8	24	1.38	1737	1811	1774	2849	3466	3158	**0.56**
B25	4.11	2.51	103	7.48	5400	3.73	1345	1402	1374	1363	1659	1511	**0.91**
B26	7.41	9.81	727	28.3	24	1.38	1688	1759	1724	2267	2758	2513	**0.69**
B27	6.53	11.90	777	26	24	1.38	2084	2173	2129	1956	2381	2169	**0.98**
B28	9.24	6.62	612	23.73	24	1.38	5023	5182	5103	6193	5920	6057	**0.84**
B29	11.16	5.06	565	8.3	8700	3.94	1715	1788	1752	2528	3076	2802	**0.63**
B30	21.57	13.10	2826	19.5	130000	5.11	3868	4032	3950	5551	6755	6153	**0.64**
B31	7.35	14.60	1073	36.8	13000	4.11	1128	1176	1152	1195	1454	1325	**0.87**
B32	10.9	15.70	1711	25	24	1.38	1585	1652	1619	2037	2478	2258	**0.72**
B33	5.95	17.70	1053	30.2	24	1.38	3935	4468	4202	4586	5137	4862	**0.86**
B34	11.78	11.70	1378	17.9	1500	3.18	3256	3394	3325	4639	5645	5142	**0.65**
B35	16.51	4.56	753	7.27	330000	5.52	2693	2808	2751	2520	3067	2794	**0.98**
B36	9.71	18.30	1777	25.5	180000	5.26	978	1020	999	1558	1896	1727	**0.58**
B37	4.01	6.33	254	18.6	24	1.38	1488	1398	1443	1941	1795	1868	**0.77**
B38	13.38	6.32	846	15.3	88000	4.94	3219	3355	3287	3536	4302	3919	**0.84**
B39	5.46	19.10	1043	26.8	24	1.38	1511	1575	1543	1788	2175	1982	**0.78**
B40	9.33	23.50	2193	38.1	24	1.38	1387	1512	1450	3118	2022	2570	**0.56**
B41	11	13.33	1466	21	37000	4.57	2691	2639	2665	5071	5191	5131	**0.52**
B42	14.12	5.57	786	26.5	330	2.52	1492	1464	1478	2454	2512	2483	**0.60**
B43	7.2	12.40	893	22.5	24	1.38	2626	2347	2487	2793	2462	2628	**0.95**
B44	10.7	21.40	2290	35.1	6600	3.82	1798	1764	1781	1938	1983	1961	**0.91**
B45	17.9	2.67	478	15.8	99000	5.00	962	944	953	2129	2179	2154	**0.44**
B46	10.85	14.30	1552	24.1	6800	3.83	1095	1074	1085	1379	1412	1396	**0.78**
B47	11.34	6.30	714	13.6	24	1.38	2194	2179	2187	3840	3427	3634	**0.60**
B48	9.26	26.10	2417	35.9	410	2.61	933	915	924	1012	1036	1024	**0.90**

University of Cape Town

Cohort B: HIV-infected patients treated with antiretrovirals: 5

Cohort B: ARV Exposed

Code	WCC CD45	CD4% WCC	CD4 abs X	CD4% Lymphs	Viral Load F	log vl	COX-1 a	COX-1 b	COX-1 Mean	ASPOLG-2 a	ASPOLG-2 b	ASPOLG-2 Mean	mt:n Ratio
B49			433	7.39	99000	5.00	1485	1475	1480	2371	2427	2399	**0.62**
B50	6.82	5.60	382	9.44	13000	4.11	765	751	758	1138	1165	1152	**0.66**
B51	7.13	7.85	560	16.2	24	1.38	951	932	942	849	869	859	**1.10**
B52	3.36	25.80	867	40.8	24	1.38	432	424	428	331	339	335	**1.28**
B53	8.91	5.65	503	8.37	30000	4.48	1401	1527	1464	1314	1497	1406	**1.04**
B54			1130	17.69	24	1.38	272	267	270	323	331	327	**0.82**
B55	15.57	6.28	978	17.2	8600	3.93	245	241	243	477	488	483	**0.50**
B56	8.15	11.50	937	21.9	24	1.38	459	451	455	561	574	568	**0.80**
B57	11.53	8.13	937	19.1	96000	4.98	851	835	843	899	920	910	**0.93**
B58	6.32	17.80	1125	38.9	24	1.38	489	480	485	399	408	404	**1.20**
B59	11.38	2.06	234	4.4	57000	4.76	630	619	625	990	1014	1002	**0.62**
B60	8	25.21	2017	38	24	1.38	557	547	552	469	480	475	**1.16**
B61	5.32	18.50	984	30.7	24	1.38	346	339	343	347	356	352	**0.97**
B62	9.03	16.90	1526	34.1	24	1.38	307	301	304	364	373	369	**0.82**
B63	5.53	21.59	1194	34.4	24	1.38	248	243	246	328	336	332	**0.74**
B64	9.79	17.40	1703	35.5	24	1.38	2074	2034	2054	1935	1981	1958	**1.05**
B65	12.47	11.40	1421	23.1	24	1.38	3393	3169	3281	3456	3510	3483	**0.94**
B66	10.37	9.92	1029	18.4	16000	4.20	1978	1940	1959	1794	1836	1815	**1.08**
B67	18.04	10.59	1911	21.31	24	1.38	3210	3149	3180	3267	3345	3306	**0.96**
B68	9.15	14.40	1318	31.3	24	1.38	1039	1019	1029	889	910	900	**1.14**
B69	9.54	14.30	1364	27.8	1200	3.08	2579	2530	2555	2586	2647	2617	**0.98**
B70			1742	26.11	24	1.38	2723	2671	2697	2578	2639	2609	**1.03**
B71	7.53	17.80	1340	25.6	24	1.38	1709	1910	1810	1451	1342	1397	**1.30**
B72	6.84	10.10	691	18.3	24	1.38	2202	2350	2276	2525	2336	2431	**0.94**
B73	6.38	16.82	1073	40	24	1.38	1261	1346	1304	1600	1480	1540	**0.85**
B74	5.63	15.42	868	32.2	24	1.38	1687	1800	1744	1444	1336	1390	**1.25**
B75	13.59	10.79	1467	21.1	14300	4.16	3167	3379	3273	3826	3539	3683	**0.89**
B76	7.07	10.30	728	30.6	24	1.38	1936	2065	2001	1679	1553	1616	**1.24**
B77	8.91	10.00	891	21.5	24	1.38	1896	2023	1960	2247	2079	2163	**0.91**
B78	5.3	15.91	843	23.4	24	1.38	1896	2023	1960	1833	1696	1765	**1.11**
B79	6.64	22.39	1487	30.1	93000	4.97	1306	1393	1350	2107	1949	2028	**0.67**
B80	6.64	15.20	1009	21.8	24	1.38	860	917	889	1330	1231	1281	**0.69**
B81	10.38	14.70	1526	38.9	24	1.38	1715	1830	1773	1400	1295	1348	**1.32**
B82	13.93	14.50	2020	30.6	24	1.38	2309	2443	2376	3798	4104	**3951**	**0.60**
B83	5.46	8.11	443	17.8	24	1.38	1378	1458	1418	2283	2467	**2375**	**0.60**
B84	8.59	16.00	1374	23.6	24	1.38	1100	1163	1132	1355	1464	**1410**	**0.80**
B85	14.16	8.01	1134	15	530	2.72	2773	2935	2854	3965	4284	**4125**	**0.69**
B86	10.47	10.51	1100	16.8	130	2.11	1977	2110	2044	2869	2655	**2762**	**0.74**
B87	8.24	10.59	873	14.9	340	2.53	2546	2717	2632	3106	2847	**2977**	**0.88**
B88	5.05	27.70	1399	40.3	24	1.38	1550	1689	1620	973	1109	**1041**	**1.56**

Cohort B: HIV-infected patients treated with antiretrovirals: 6

Cohort B: ARV Exposed

Code	WCC CD45	CD4% WCC	CD4 abs X	CD4% Lymphs	Viral Load F	log vl	COX-1 a	COX-1 b	COX-1 Mean	ASPOLG-2 a	ASPOLG-2 b	ASPOLG-2 Mean	mt:n Ratio
B89	3.29	3.07	101	5.66	24	1.38	1123	1198	1161	1400	1295	**1348**	**0.86**
B90	6.09	11.22	683	22.6	24	1.38	1780	1899	1840	2502	2315	**2409**	**0.76**
B91	17.57	15.09	2651	25.4	24	1.38	2403	2565	2484	3744	3492	**3618**	**0.69**
B92	9.45	9.45	893	13.2		#NUM!	2358	2514	2436	2316	2143	**2230**	**1.09**
B93	11.75	11.00	1293	15.8	230	2.36	1297	1365	1331	2347	2172	**2260**	**0.59**
B94	11.54	0.73	84	11.8	24	1.38	1846	1970	1908	4010	3710	**3860**	**0.49**
B95	14.04	3.88	545	27.7	24	1.38	1833	1956	1895	3493	3232	**3363**	**0.56**
B96	4.82	16.20	781	23.4	140	2.15	734	784	759	1248	1154	**1201**	**0.63**
B97	10.84	14.80	1605	39.1	24	1.38	1062	1134	1098	1727	1598	**1663**	**0.66**

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Cohort C: Presumed HIV negative controls: 1

Cohort C: Presumed HIV negative Demographic Data

Code	Hospital Number	Sex	Weight(kg)	Length(cm)	Age (Mo)	Z-height	Z-weight	Z W for H	Date	Procedure	Anaesthetic
C1	13354014	f	13	90	25.56	1.26	0.58	0.05	23/2/2005	Bleomycin Inj	VIMA/local infiltr
C2	13382650	f	15.3	91	38.73	-1.2	0.36	1.47	23/2/2005	Celestone inj	VIMA/local infiltr
C3	82457227	f	27.5	130	112.98	-0.71	0.55	0.17	23/2/2005	Keloid remova	VIMA/local infiltr
C4	11486602	f	11.85	85	21.93	0.4	0.41	0.32	2/3/2005	Adenoidecton	VIMA/Propofol/
C5	85253011	m	22.5	120	79.01	0.12	0.17	0.13	2/3/2005	Adenoidecton	VIMA/Propofol/
C7	13314851	m	23	115	81.77	-1.13	0.16	1.3	2/3/2005	Adenoidecton	VIMA/Propofol/
C8	89227771	m	10.55	87	22.4	0.2	-1.35	-1.9	16/3/2005	Tonsills/Grom	VIMA
C9	86083805	m	13.65	104	59.46	-1.23	-2.34	-2.18	16/3/2005	Adenoids/tons	VIMA
C10	13253075	m	15.7	105	50.85	0.07	-0.75	-0.97	16/3/2005	Adenoids/tons	VIMA
C11	86706470	f	16	105	50.85	0.41	-0.21	-0.5	16/3/2005	Adenoids/tons	VIMA
C12	87542452	f	14.8	100	42.08	0.52	-0.18	-0.42	23/3/2005	Adenotonsille	VIMA
C13	87913281	m	13.6	98	42.15	-0.29	-1.19	-1.22	23/3/2005	Adenotonsille	VIMA
C14	83271361	f	20.3	117	107.69	-2.32	-1.86	-0.89	23/3/2005	Adenotonsille	VIMA
C15	13789722	m	20.3	113	57.92	0.93	0.84	0.34	23/3/2005	Adenotonsille	VIMA
C16	85617314	m	12	108	72.67	-1.73	-3.75	-3.87	6/4/2005	Bleomycin Inj	VIMA
C17	13815998	m	17	108	60.25	-0.45	-0.81	-0.66	6/4/2005	Lipoma poplit	VIMA
C18	13950282	m	18	120	57.39	2.54	-0.12	-2.3	6/4/2005	Skin Graft po:	VIMA
C19	13188321	f	8.6	74	12.65	-0.38	-1.04	-0.91	6/4/2005	Removal of e:	VIMA
C20	13205547	m	6.9	63	10.51	-1.96	-2.17	-0.86	6/4/2005	Removal of e:	VIMA
C21	13405014	F	12	88	31.9	-0.82	-1	-0.44	13/4/2005	Adenotonsille	VIMA
C22	85719235	f	14	103	69.45	-2.15	-2.35	-1.52	13/4/2005	Adenoidecton	VIMA
C23	13560503	f	10	98	36.37	1.03	2.97	3.22	13/4/2005	Adenotonsille	VIMA
C24	85832582	m	16	104	64.39	-1.76	-1.55	-0.58	13/4/2005	Adenotonsille	VIMA
C25	89270367	m	39	128	93.1	0.43	3.26	4.02	11/5/2005	Adenotonsille	VIMA/ propofol
C26	89269153	f	21	113	69.38	-0.06	0.6	0.86	11/5/2005	Adenotonsille	VIMA/ propofol
C27	82565920	f	32	136	112.52	0.23	0.28	0.21	11/5/2005	Adenoidecton	VIMA/ propofol
C28	86278678	f	21	113	70.3	-0.16	0.54	0.86	11/5/2005	Adenotonsille	VIMA/ propofol

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Cohort C: Presumed HIV negative controls: 2

Cohort C: Presumed HIV negative Light Cycler Data

Code	COX-1 a1	COX-1a2	COX-1 b1	COX-1b2	COX-1 mean	ASPOLG-2 a1	ASPOLG-a2	ASPOLG-2 b	ASPOLG-b2	ASPOLG-2 mean	mt:n DNA ratio
C1	5833	5746	6615	6517	6178	4970	4913	5029	4971	4971	**1.24**
C2	5421	5340	4785	4714	5065	4894	4838	5422	5360	5129	**0.99**
C3	3979	3920	3869	3811	3895	3254	3217	3263	3225	3240	**1.20**
C4	7687	7572	7196	7089	7386	6027	5958	6480	6404	6217	**1.19**
C5	4532	4465	4654	4584	4559	2904	2871	2872	2839	2872	**1.59**
C7	2742	2701	2787	2745	2744	2715	2684	2719	2688	2702	**1.02**
C8	4462	4396	4985	4911	4689	3984	3938	4492	4441	4214	**1.11**
C9	4541	4473	4283	4219	4379	3359	3320	3105	3069	3213	**1.36**
C10	4434	4434	4501	4501	4468	3579	3320	3359	3320	3359	**1.33**
C11	5090	5014	5254	5176	5134	3263	3226	3210	3173	3218	**1.60**
C12	4627	4558	4729	4659	4643	4326	4276	3821	3777	4050	**1.15**
C13	1250	1250	1362	1362	1306	1709	1709	1948	1948	1829	**0.71**
C14	4620	4372	4518	4276	4447	4412	4614	4098	4286	4353	**1.02**
C15	5043	4772	4269	4040	4531	5019	5250	4735	4953	4989	**0.91**
C16	4058	3840	3880	3672	3863	4259	4454	3930	4110	4188	**0.92**
C17	3985	3771	4153	3930	3960	4084	4272	3955	4136	4112	**0.96**
C18	5482	5187	4763	4507	4985	5458	5708	5045	5276	5372	**0.93**
C19	1918	1918	2091	2091	2005	2338	2338	2665	2665	2502	**0.80**
C20	3492	3305	3758	3556	3528	3242	3391	3232	3380	3311	**1.07**
C21	4315	4983	4311	4079	4422	5073	5306	4169	4361	4727	**0.94**
C22	4700	4448	4459	4219	4457	3652	3820	3300	3452	3556	**1.25**
C23	3476	3289	3402	3219	3347	3312	3464	3168	3314	3315	**1.01**
C24	1748	1654	1748	1654	1701	1102	1261	1206	1152	1180	**1.44**
C25	3567	3376	3346	3166	3364	2810	2939	2793	2921	2866	**1.17**
C26	3318	3140	3845	3638	3485	2874	3006	2920	3054	2964	**1.18**
C27	4006	3308	3904	3224	3611	2437	2698	2443	2705	2571	**1.40**
C28	3599	2971	3439	2840	3212	2757	3052	2834	3138	2945	**1.09**

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- Author Contribution Requirements
- General Guidelines
- Journal Categories
- Title Page
- Abstract
- Text
- Disclosures/Conflict of Interest
- Acknowledgements
- References
- Tables
- Figure Captions
- Figures
- Color
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  2. Fiechtner M, Ramp J, England B, Knudson MA, Little RR, England JD, et al. Affinity binding assay of glycohemoglobin by two-dimensional centrifugation referenced to hemoglobin Alc. Clin Chem 1992;38:2372–9.

- Abstract:

  3. Hortin GL, King C, Kopp J. Quantification of rhesus monkey albumin with assays for human microalbumin [Abstract]. Clin Chem 2000;46:A140-1.

- Editorial:

  4. Demers LM. New biochemical marker for bone disease: is it a breakthrough? [Editorial]. Clin Chem 1992;38:2169–70.

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- Letter to the Editor:

  5. Davey L, Naidoo L. Urinary screen for acetaminophen (paracetamol) in the presence of N-acetylcysteine [Letter]. Clin Chem 1993;39:2348–9.

- Book Chapter:

  6. Rifai N, Warnick GR. Lipids, lipoproteins, apolipoproteins, and other cardiovascular risk factors. In: Burtis CA, Ashwood ER, Bruns DE, eds. Tietz textbook of clinical chemistry and molecular diagnostics. 4th Ed. St. Louis: Elsevier Saunders 2006:903-81.

- Thesis:

  7. Haughton MA. Immunonephelometric measurement of vitamin D binding protein [MAppSci thesis]. Sydney, Australia: University of Technology, 1989:87pp.

- Books and Monographs (serial volumes should be treated as journals):

  8. Harrell FE Jr. Regression modeling strategies. New York: Springer; 2001.

  9. Bailar JC III, Mosteller F, eds. Medical uses of statistics, 2nd Ed. Boston: NEJM Books; 1992:449pp.

- Publish-Ahead of Print:

  10. Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.

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  11. Castelli WP. Lipids, risk factors and ischaemic heart disease. Atherosclerosis 1996;124 Suppl:S1-9.

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- Internet Source:

12. American Association for Clinical Chemistry. AACC continuing education. http://www.aacc.org/services/default.stm (Accessed November 1998).

Tables

Tables are considered text. They should appear embedded as part of the submission directly following the reference section. Each table should appear on an individual page. Manuscripts will be returned to authors if the tables are uploaded as separate files.

Each table should be clearly labeled and numbered consecutively with Arabic numerals (Table 1, Table 2, etc.). The abbreviations used must be defined. As with the text, SI units must be used; however, conventional units may be added in parentheses. Conversion factors may be added as footnotes. Indicate explanatory footnotes with superscript lowercase italic letters in alphabetical order.

Tables should not include parts. Tables with parts (Table 1A, Table 1B, etc.) will be returned to authors for correction.

Table captions should be limited to 60 words.

Each column of an individual table must have a heading and be clearly defined. Indicate explanatory footnotes with superscript lowercase italic letters in alphabetical order.

Color tables will be considered for publication. As in the case with color figures, the costs of publishing color illustrations will be borne by the author. Costs are $1500 for the first color table and $500 for each subsequent color table, or part thereof. Please note the author agreement to pay in the cover letter.

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Large, complex, or tables that include graphic elements should be submitted as figures or as supplemental data for online publication only. The Editor may choose to make any table supplemental as he/she deems necessary.

Figure Captions

Figure captions and sub-captions must be listed together on an individual page directly following the tables or the references if submission does not include tables. Each figure requires a figure caption and should be clearly labeled (Figure 1, Figure 2, etc.). Multi-panel figures; figures with parts (a, b, c, etc.), should be distinctively labeled (Figure 1A, Figure 1B, Figure 2A, etc.).

Figure captions should be limited to 60 words.

If a figure has been published previously, acknowledge the original source in the figure caption and submit written permission from the copyright holder to reproduce the figure as supplemental data.

Figures

All figures must be uploaded separately as image files in Tagged Image File Format (.TIFF) or Encapsulated Postscript (.EPS). Microsoft Power Point (.PPT) is also acceptable; however, the graphics must contain embedded fonts with one image per slide, one slide per file. Each image should have a resolution of 600 dots-per-inch (dpi). More detailed specifications can be found on our print publication vendor's page at CADMUS Digital Art Support.

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The submission system is designed to retrieve the manuscript file and separate image file(s) and combine or merge the files into an individual Portable Document Format (.PDF) for reviewing purposes. It is the responsibility of the author to verify the quality of the image file(s) remains after the conversion to PDF. It may be necessary to alter the individual image file(s) to ensure the content is not lost or misconstrued in the merged PDF.

Submissions will be returned to authors if the figures are embedded within the manuscript file and not uploaded separately, or the figures have become eligible in the merged PDF.

Do not place unnecessary graphics, such as borders, in or around your figure. Pay particular attention to the quality of the lines, symbols, and patterns. Published figures are reduced to 1 column (85mm) or 2 columns (176 mm) in width. If, at that width, the figure symbols or lettering are not clear, you will need to increase the font size. Letters should be 8/10 points when reduced, subscripts no less than 6 point. Initial capitals and lower case letters and a medium (not bold) sans serif font is required. European PI, Helvetica, Mathematical PI, Times Roman, and Symbol fonts are Type 1 PostScript fonts supported by our printer for the creation of digital art figures.

If publication quality electronic figure files cannot be provided, acceptable publication prints are:

- Halftone: glossy prints
- Line drawing: glossy prints, laser prints on coated (nonabsorbent) laser-printer paper, or drawings in black India ink on tracing paper.
- Color: glossy prints, laser prints on coated (nonabsorbent) laser-printer paper

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[Halftone: Image with blocks or portions that are gray opaque, such as a photograph; Line drawing: Image is in black and white only, such as a line graph; Color: Images where color is necessary to the meaning of the image]

The x-axis and y-axis should be clearly labeled, and the units of measurements given. This includes using decimal points instead of commas, italicizing species or gene symbols, and using U.S. English.

Color

The costs of publishing color illustrations in print will be borne by the author. As with tables, costs are $1500 for the first figure and $500 for each subsequent figure, or parts of figures. Upon acceptance, authors will be billed for the color costs unless a request to print figures in black and white is received via cover letter or e-mail. Color versions of figures may be provided for publication in the on-line Journal at no extra charge. Please note your color preference and/or your agreement to pay for color printing in your cover letter.

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Supplemental Data

Figures or tables too large for print, manuscript material that exceeds the limitation for the specific submission type, or appendices should be submitted for online publication only. These files should be marked and uploaded separately as supplemental files during submission, and should be referenced within the text as supplemental data (i.e., "See Supplemental Data Table 1", "See Supplemental Data Figure 1", etc.). Supplemental files

must be labeled consecutively (i.e., Supplemental Data Table 1, Supplemental Data Table 2, Supplemental Data Figure 1, Supplemental Data Figure 2, etc.).

Captions for the supplemental data should be uploaded as a separate supplemental file or embedded within the supplemental data/table/figure file. Do not include the supplemental captions in your manuscript file.

Please be advised, supplemental data are permitted for Article, Brief Communication, Inspiring Minds, Mini-Review, Review, and Special Report submissions only. Manuscripts of all other types with supplemental data will be returned to authors.

Supplemental data will be reviewed as part of the manuscript and will be evaluated for its importance and relevance; however, it should not appear in the "Peer-reviewed PDF" file created by the submission system.

If accepted and posted online, the supplemental data will be referenced in the text of the print article directing readers to the URL of the Web site.

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Faculty of Health Sciences

MMed/MPhil Part III (minor dissertation)

Guidelines for candidates, supervisors and examiners

Minimum Requirements for Dissertations for MMed and MPhil for Subspecialities Degrees

Following extensive discussion with Heads of Divisions, Dr S Kalula and Prof S Kidson recommend the following minimum criteria for dissertations for MMed and MPhil (subspeciality) degrees:

The MMed minor dissertation (or the MPhil dissertation in the case of sub-specialities) is one of three examination components of the MMed/MPhil degree. This minor dissertation carries one third of the weight of a full master's dissertation in terms of its credit weighting. The dissertation must be a study containing the results of an analytical, quantitative, or epidemiological study carried out by the candidate (for certain disciplines, the candidate may chose instead to do a qualitative study, an audit cycle or a formal systemic review). A case report is not acceptable for the dissertation.

The dissertation must be the result of independent work of the candidate conducted under the guidance and direction of a supervisor(s) and should demonstrate evidence of an ability to undertake research, to adequately interpret results and to comprehensively and critically review the relevant literature. Although the findings of the research need not

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necessarily be original, they must be seen to advance scientific understanding. The topic and scope of research will depend on the particular disciplines and must be agreed upon in consultation with the supervisor(s).

Research protocol

Candidates intending to register for the MMed/MPhil Part III are required to submit a full research protocol for approval to their respective Departmental Research Committee (DRC). The candidate must also obtain FHS UCT Ethics approval prior to conducting their research. This full research protocol (together with a copy of the ethics approval letter) must be submitted to the postgraduate administration for approval by the Board of the Faculty of Health Sciences, prior to commencement of the research. For most disciplines, submission of the research protocol should be made no later than the end of year 2. The research protocol should outline the scope and content of the dissertation and must include the title of the proposed dissertation, name of the supervisor(s) and their brief curriculum vitae.

Submission of dissertations

On completion, the dissertation should be submitted to the Faculty Postgraduate Officer. The candidate should inform the Faculty Officer one month in advance of the intention to submit.

Submission deadlines:

1. March 15th for June graduation
2. August 15th for December graduation

Supervisors will be requested by the Faculty Postgraduate Officer to submit a letter supporting submission. This letter should be supplied by the primary supervisor. If this

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supervisor is external, the internal supervisor must be kept informed at every stage of the process. Specific submission requirements may be set by individual disciplines.2

Note on fees: To avoid attracting fees, dissertations need to be submitted before the beginning of the first quarter (first day of academic year), and before the start of the second semester (mid July) to qualify for a 50% fee rebate.

Supervisors

One cannot overemphasize the importance of identifying a dissertation supervisor as early as possible. The supervisor should be an individual who can relate to the candidate's research project, be available for frequent and regular discussion and advice, and someone with whom the candidate can develop a good working relationship. Where specialised equipment and/or laboratory work is required for the study, the supervisor should assist in facilitating such access to such facilities. Supervisors may assist candidates in developing scientific communication skills but they are not required to do detailed editing or correction of spelling, grammar, or style. They may refer candidates to the UCT Writing Centre for this purpose.

The primary supervisor may be based outside the candidate's home department, faculty or university. In such a case, an internal (or secondary) supervisor will be required in addition to the primary supervisor, to serve as a guide and link to discipline-specific procedures. Primary supervisors retain responsibilities to the candidate and the university until the dissertation process is complete.

Please note: in order to assist a candidate with a master's research topic the supervisor needs to hold a master's degree or higher, or have relevant research experience. If the primary supervisor does not hold a higher degree or equivalent (such as a Fellowship of

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The College of Medicine of South Africa), then a secondary supervisor, who has a higher degree will need to be appointed in addition to the primary supervisor.

Candidates are strongly encouraged to publish the study with the supervisor(s) as co-author(s). This may require work beyond the graduation date. Such arrangements should be discussed and documented in advance.

2 For Public Health Medicine and Occupational Medicine the dissertation must be submitted for examination at least *4 months* prior to the deadline for registration for the examinations of the relevant College. This is in order to ensure that a final examination mark for the dissertation can be submitted by the candidate to the College of Medicine of South Africa (CMSA) at the time of registration as required by CMSA examination regulations.

The dissertation

Submission of the dissertation should satisfy the following criteria:

1. The title page should contain the candidate's name, dissertation title and the name of the university.

It must also state the degree, e.g. Master of Medicine (MMed) in Public Health Medicine, Occupational Medicine, Family Medicine, Surgery, etc. The title page should also include a statement to the effect that the research report is based on independent work performed by the candidate and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. It must also state that this work has not been published *prior to registration* for the abovementioned degree.

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2. The body of the dissertation, which must be structured in 4 parts, should include the following:

Part A: The *protocol* (as approved by the Departmental Research Committee and Faculty Research Ethics Committee). The protocol should not exceed 4000 words.

Part B: A *structured literature review* appropriate to the subject matter and methods of the dissertation. The literature review must, amongst other things, show that the student is sufficiently acquainted with the relevant literature and is able to perform a critical appraisal and, if appropriate for the topic, show a good understanding of evidence-based medicine. The review should be between 3 000 and 4 000 words. A suggested structure for the literature review is as follows:

a) Objectives of literature review

b) Literature search strategy, including inclusion and exclusion criteria

c) Quality criteria - some leeway will be allowed here, as candidates will vary in their ability to appraise studies. This will also vary with the nature of the dissertation.

d) Summary or interpretation of literature

e) Identification of gaps or needs for further research

f) References (which will overlap with but will not be the same lists as in the journal article and protocol)

Part C: The results of the study must be presented in the form of a *manuscript* of an article for a named peer reviewed journal, meeting all the requirements set out in the "Instructions for Authors" of that journal, including the word count and referencing style. (Unless specially motivated, the journal chosen will need to allow for *at least* 3000 words excluding abstract, tables, figures and references). The "Instructions to Authors" of the

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journal must be appended. The journal chosen for publication must be appropriate to the subject matter of the dissertation and accredited by the Department of Education or listed in the citation index of the Institute for Scientific Information (ISI). Important note: the candidate need not have submitted the article, not is the acceptance of the article and requirement for passing the degree. The norm of practice is to publish the study with the supervisor(s) as co-author(s) and candidates are strongly encouraged to submit their manuscript either before or after examination of the mini-dissertation.

Part D: All *supporting documents* including:

• Questionnaire/data capture instrument

• Consent forms and any related participant information sheets

• Technical appendices, including, if considered necessary, any additional tables not included in the main manuscript for the examiner to have available. These should be accompanied by a brief narrative.

• Official Ethics approval letter from the Faculty Research Ethics Committee

3. The article does *not* have to be submitted to the journal in order to meet academic requirements.

4. A candidate must submit 2 copies of the dissertation in temporary binding, and an electronic copy on compact disc in a universally readable format (e.g. pdf).

Examiners

The full dissertation will be submitted for examination through the Postgraduate office of our Faculty to two external examiners (nominated by the supervisors and HOD).Three examiners will be nominated, two of which are invited to examine, and one held as an alternate. All examiners must be external to UCT. These nominations are circulated to the

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Faculty Dissertation Committee. It is the *supervisor's (or co-supervisor's)* responsibility to submit names of potential examiners to the Faculty Officer when the candidate is ready to submit. The examiners will be well briefed regarding the specific requirements and criteria for submission and examination of the mini-dissertation. Such criteria will clearly explain the difference between the mini dissertation and a Master's degree by dissertation alone. Details required for each examiner are: academic qualifications, postal and/or physical address, telephone and fax numbers and e-mail address, and one paragraph description of their standing in the relevant field (drawn from their CV if need be.) *The candidate may not be informed of the identity of the examiners*. After the outcome of the mini dissertation has been finalized, the examiners' identities are made known if the examiners have indicated that they do not object to this.

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