



## **INTER AND INTRA-LABORATORY VARIABILITY OF CD4: A PRAGMATIC ANALYSIS**

By

Dr Ganizani Mlawanda

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Supervisor: Professor Paul Rheeder

Co-Supervisor: Dr Jacqui Miot

# DECLARATION

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I, Dr Ganizani Mlawanda, hereby declare that the dissertation which I hereby submit for the degree Master of Science in Clinical Epidemiology at the University of Pretoria is my own work and has not previously been submitted by me for a degree at another university.

\_\_\_\_\_  
Dr Ganizani Mlawanda

\_\_\_\_\_ 2011  
Date

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# ABSTRACT

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**Background:** Measuring CD4 levels is the key laboratory investigation for decision making when initiating HAART, a tertiary prevention measure to reduce HIV/AIDS mortality and morbidity. Inherent biological and analytical variability is common during CD4 enumeration. We cannot control biological variation but how significant is analytical variation to clinical decision making.

**Objectives:** To quantify inter and intra laboratories analytical variation of CD4 counts and percentages and to determine the degree to which time lapse after sample collection contributes to the analytical coefficient of variation (CV%). To estimate the extent of disease misclassification due to CD4 variability if  $CD4 < 350 \text{ cells/mm}^3$ .

**Setting:** This study was conducted at the HIV clinics of RSSC Hospital, a sugar-cane estate health institution located on the north-east of Swaziland, in Lubombo district, the worst affected by HIV/AIDS in Swaziland. The laboratories involved were Lancet, Good Shepherd (GSH) and National Reference (NRL) laboratories.

**Study design and method:** An analytical diagnostic, cross-sectional (observational) study was used in this study. Using a convenience sampling technique and after obtaining consent from participants, blood was collected in EDTA tubes and sub-divided into three samples, each for Lancet, GSH and NRL. The samples were further split into two at each respective laboratory, one of which was run at 12 hours and the other at 24 hours from the time of sample collection.

**Main outcome measures:** Student t-test; analytical coefficient of variation (CV%); Bland and Altman (BA) method bias and limits of confidence; BA plots and percentage difference plots; concordance correlation, Pearson and Kappa coefficients; McNemar test for comparison of paired proportion.

**Results:** Fifty three participants consented for participation and of these twenty eight participants were male. The mean CD4 was  $373.4 \text{ cells/mm}^3$  for Lancet,  $395.9 \text{ cells/mm}^3$  for NRL and  $439.2 \text{ cells/mm}^3$  for GSH and subsequent paired t-test revealed some inherent variability. The CV% for CD4 count was 3.5%, 8.4% and 20.1 whilst bias was 7.0, 13.5 and 8.2 for NRL, Lancet and GSH respectively. CD4% had even stronger CV% for all three laboratories. Inter-laboratory bias for Lancet/NRL was -31.5; -64.3 for Lancet/GSH and -38.2 for NRL/GSH at 12 hours for CD4 count with only Lancet/GSH having a clinically interchangeable limit of agreement. At 24 hours, the trends were similar, possibly confirming stability of CD4 between 12 and 24 hours. An assessment of disease misclassification at HAART initiation threshold was performed. The agreement was 81.1% for Lancet/NRL, 88.7% for Lancet/GSH and 77.4% for NRL/GSH corresponding to Kappa values of 0.64, 0.77 and 0.55 respectively. McNemar test for paired proportions revealed that there were no differences between the laboratories when it came to initiating HAART.

**Conclusions:** whilst intra-laboratory variability is minimal, there is some significant inter-laboratory variation of CD4 count and CD4% at the laboratories used in Swaziland. Swaziland should ensure standard SOPs, on-going training and continuous quality improvements for all national laboratories and ensure standards are on par with international recommendations. The national HIV guidelines should possibly enforce two different CD4 counts in decision making to reduce systematic errors. Meanwhile, clinicians should continue to use their clinical judgment in cases of suspicious CD4 count results.

**Key Words:** CD4 variability; agreement; inter-laboratory; intra-laboratory

# CONTACT DETAILS

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**Principal Investigator:** Dr Ganizani Mlawanda

MSc Clinical Epidemiology candidate – School of Health Systems and Public Health  
University of Pretoria  
Faculty of Health Sciences  
School of Health Systems and Public Health  
5th Floor, HW Snyman Building North  
31 Bophelo Road  
Gezina  
Pretoria  
0031  
Email: [ganizanim@rssc.co.sz](mailto:ganizanim@rssc.co.sz); [mlawandag@yahoo.co.uk](mailto:mlawandag@yahoo.co.uk)

**Supervisor/Promoter:** Professor Paul Rheeder

University of Pretoria  
Faculty of Health Sciences  
School of Health Systems and Public Health  
5th Floor, HW Snyman Building North  
31 Bophelo Road  
Gezina  
Pretoria  
0031  
Email: [Paul.Rheeder@up.ac.za](mailto:Paul.Rheeder@up.ac.za)

**Co-Supervisor/Co-promoter:** Dr Jacqui Miot

University of Pretoria  
Faculty of Health Sciences  
School of Health Systems and Public Health  
5th Floor, HW Snyman Building North  
31 Bophelo Road  
Gezina  
Pretoria  
0031  
Email: [jacqui.miot@gmail.com](mailto:jacqui.miot@gmail.com)

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# ABBREVIATIONS

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WHO:	World Health Organization
HIV:	Human Immunodeficiency Virus
HAART:	Highly Active Antiretroviral Therapy
AIDS:	Acquired Immune Deficiency Syndrome
CD:	Cluster of differentiation
CD4:	Cluster of differentiation 4. Absolute CD4 count
CD4%:	Cluster of differentiation percentage
%CD4 DIFFERENCE PERCENTAGE:	difference of CD4 count
RCT:	Randomised Controlled Trial
RSSC:	Royal Swaziland Sugar Company
GSH:	Good Shepherd Hospital
NRL:	National reference laboratory
EDTA:	Ethylenediaminetetraacetic acid
BA:	Bland and Altman
Hr:	hour
Hrs:	hours
95% CI:	95% Confidence Interval
H <sub>0</sub> :	Null hypothesis
P-value:	$\alpha$ value = 0.05. A p-value of 0.05 was used throughout the study
sd error:	standard error
SWILK:	Shapiro-Wilk test for assessing normality
SOPs:	Standard operating procedures
Lab:	Laboratory

# CHAPTER: 1: LITERATURE REVIEW

## 1.1 INTRODUCTION

On the 1<sup>st</sup> of December 2009, the World Health Organisation (WHO), using a “Rapid Advice” document, officially made available the latest recommendations for initiating Antiretroviral Therapy (ART) in Human Immunodeficiency Virus (HIV) infected patients, at a CD4 count of 350cells/mm<sup>3</sup>.<sup>1</sup> The new recommendation relies primarily on CD4 cell count.

The CD4 count is the standard test to assess prognosis for progression to Acquired immune Deficiency Syndrome (AIDS) or death, to formulate the differential diagnosis in a symptomatic patient and to make therapeutic decisions regarding Highly Active Anti-Retroviral Therapy (HAART). A CD4 count below 350cells/mm<sup>3</sup> is the new indication for initiating HAART. The alternative but less reliable option is clinical staging. At high CD4 values, the clinical staging has very low predictive value for decisions whether to initiate treatment. CD4 count enumeration is therefore the most useful parameter in HIV management. Ensuring quality and highly reliable CD4 counts has become even more significant now that HAART is being initiated at higher CD4 counts, 350cells/mm<sup>3</sup>, hence less clinical signs.

Despite the vast importance of CD4 counts in HIV management, considerable between-laboratory and intra-laboratory variability may occur. In Swaziland, there have been various undocumented reports on gross inter- and intra-laboratory discrepancies in CD4 count results at various HIV clinics, probably significant enough to affect clinical decision making. In line with these potential clinical and laboratory problems, this study seeks to shed more light on how well our CD4 counts reflect the truth, whether patients can freely use any laboratory located closer to them confidently, to address clinicians’ worries with CD4 results and finally, a general comparison with those from an internationally accredited reference laboratory.

Using an analytical diagnostic, cross-sectional (observational) study design and a representative sample of CD4 results for all clinical stages, this study attempted to quantify the variations associated with CD4 enumeration.

## 1.2 HISTORICAL BACKGROUND

### 1.2.1 Global HIV / Aids: the extent of the problem

The HIV / AIDS pandemic continue to devastate the global health picture. Worldwide, there were an estimated 33million (30million – 36 million) people living with HIV as at end of 2007. Two thirds of people living with HIV are in sub-Saharan Africa, although this region contains little more than 10% of the world's population.<sup>2</sup> Several countries in sub-Saharan Africa report infection rates of 30 %, especially in urban areas.<sup>3</sup> Southern African countries have the highest prevalence compared to other regions. Swaziland had an HIV prevalence of 38% as of end of 2007.<sup>2</sup> Thus HIV /AIDS is a global problem and Swaziland is the worst affected in the world.

### 1.2.2 HIV / AIDS pandemic: the impact

The consequences of the HIV / AIDS pandemic are diverse. Since the beginning of the HIV / AIDS pandemic, more than 15million Africans have died from AIDS. During 2007 alone, an estimated 1.5million adults and children died as a result of AIDS in sub-Saharan Africa.<sup>4</sup> Most of those affected are the young, economically productive age group. Apart from the high mortality, the health, education, economic and social sectors are all affected. In addition households, life expectancy, enterprise and workplaces are severely affected.<sup>5</sup> Children are not spared either, with a huge number of orphans and child headed families. Finally, there are yet unknown future consequences.<sup>5</sup>

### 1.2.3 Prevention of HIV

Primary, secondary and tertiary prevention measures have been implemented globally with different success rates. Abstinence, being faithful, condom use, delayed sexual debut, vaccines, male medical circumcision, needle exchange programs, microbicides, pre and post exposure prophylaxis and therapy are some of the primary prevention measures being implemented. The success of these interventions has been variable in different regions.<sup>6</sup> In Swaziland, the incidence of HIV has remained extremely high despite these various primary interventions.

On a positive note, HAART, a tertiary rehabilitation measure, greatly reduces the devastating consequences of HIV. Mortality rates and morbidity have decreased significantly in 20 years by 50% and now AIDS is considered a chronic and manageable disease.<sup>7</sup>

Thus although HIV prevention measures are not being as effective as anticipated, HAART has greatly changed the natural history of HIV.

### 1.2.4 Role of HAART

There has been a hive of activities to mitigate the HIV pandemic and its consequences. The pharmaceutical industry's contribution, based on the research for more active new drugs, has been pivotal.<sup>8</sup> HAART reverses the adverse consequences of HIV /AIDS. Its benefits are virological, immunological, clinical and epidemiological. Virologically, HAART directly leads to a reduction in the viral load to undetectable levels. Immunologically, it brings immune reconstitution that is both qualitative and quantitative. Clinically, it brings prolongation of life and improves quality of life. Epidemiologically, it leads to reduced transmission of HIV.<sup>8</sup> HAART has been found to uniformly

significantly reduce the consequences of HIV regardless of the region. Thus HAART is the cornerstone of mitigating the devastating impact of HIV / AIDS.

### **1.2.5 HAART and CD4: the association**

The CD4 count is critical for determining the clinical stage of HIV infection, for deciding when to start antiretroviral therapy (HAART), for evaluating the efficacy of treatment, and for changing the medications when necessary.<sup>10</sup> The CD4 count is the standard test to assess prognosis for progression to AIDS or death, to formulate the differential diagnosis in a symptomatic patient and to make therapeutic decisions regarding Highly Active Anti-Retroviral Therapy (HAART). A CD4 count below 350cells/mm<sup>3</sup> was recommended as the new indication for initiating HAART.<sup>1</sup>

### **1.2.6 What is CD4: definition and roles?**

The CD4 T lymphocytes are a subpopulation of the lymphocytes also known as T helper cells. They are coordinators of the body's immune response, for example providing help to B cells in the production of antibodies, as well as in augmenting cellular immune response to antigens. The "CD" or cluster of differentiation is a protein expressed on the surface of the cells of the hematopoietic system. The expression of these proteins is used in lymphocyte nomenclature. CD4 T lymphocytes occupy the central position in regulating immune functions.<sup>9</sup>

CD4 T lymphocytes are the primary targets of HIV. The relentless destruction of CD4 T lymphocytes by HIV, either directly or indirectly, results in the loss of HIV-specific immune response, recall antibody response and, finally, non-specific immune response in the AIDS stage.<sup>3</sup>

Normal adult CD4 count reference ranges vary by laboratory; however, ranges are generally within 500 -1500cells/mm<sup>3</sup>. Large individual variability in measurement reflects that CD4 count is based on three variables: white cell count, lymphocytes percentage and percentage lymphocytes that are CD4+ (CD4%).<sup>8</sup>

Measurement of peripheral blood CD4 T lymphocytes is the most important laboratory assay for evaluation of the degree of immune suppression and stage of disease as well as monitoring of patients with HIV. The CD4 count is critical for determining the clinical stage of HIV infection, for deciding when to start antiretroviral therapy (HAART), for evaluating the efficacy of treatment, and for changing the medications when necessary.<sup>10</sup> While clinical criteria are also recommended in resource limited settings, one study noted that clinical events do not fully predict immunological status.<sup>11</sup> When clinical criteria alone are used, some patients with Stage I and Stage II disease and severe immune suppression will not receive the treatment they need, while others with Stage III and IV disease may still have high CD4 T cell counts and the start of antiretroviral therapy might be delayed.<sup>11</sup> At high CD4 values, there are few minor clinical complications whereas at low CD4 the associated complications are multiple and more severe. Thus clinicians are more likely to diagnoses HIV complications associated with low CD4 than those associated with high CD4. Likewise, patients are likely to present with complications associated with low CD4 than those which occur at high CD4 values, mostly because of the differences in severity.(see Appendix 6: Correlation of complication of HIV and CD4 count) Thus CD4 count enumeration becomes the most useful parameter in HIV management and, of late, ensuring quality reliable CD4 counts is even more important considering that HAART is being started at high CD4 counts (hence less clinical signs), of 350cells/mm<sup>3</sup> versus

200cells/mm<sup>3</sup> which was originally recommended in older guidelines (see Appendix 4, 5,6). Thus all HIV treatment decisions should be based upon the CD4 count, where it is available.

### **1.2.7 CD4 count and HIV: the association**

Following HIV infection, there is an abrupt decline in CD4 count. After recovery from acute infection, the CD4 count can return to normal range (close to pre-infection baseline) or can be low. Thereafter, the CD4 gradually decreases reaching <200cells/mm<sup>3</sup> in an average of eight to ten years. When the CD4 count reaches 350cells/mm<sup>3</sup>, HAART is recommended. When it has dropped below 200cell/mm<sup>3</sup>, Cotrimoxazole prophylaxis is recommended. WHO recommends that HAART be started at a CD4 of 350cells/mm<sup>3</sup>. These recommendations are supported by moderate quality of evidence for critical patient and public health outcomes from one random controlled trial (RCT), the CIPRA-HT001 (a single-centre trial in Haiti) and one post hoc analysis nested in a RCT, the SMART trial (a multi-centre study in 33 predominantly high income countries).<sup>12</sup> After initiating HAART, there is biphasic increase in CD4 count of about 50-120cells/mm<sup>3</sup> in first 3 months (thought to be due to redistribution of memory CD4 cells from lymphoid tissue) followed by average increase of 2-7cells/mm<sup>3</sup> /month via expansion of naïve CD4 cell populations.<sup>13</sup>

### **1.2.8 CD4 count variability**

CD4 measurement is subject to biological or analytical/laboratory variations. Biological factors that influence CD4 cell counts include seasonal and diurnal variations (lowest at 12:30 pm, highest at 8:30 pm), surgery, viral infections, tuberculosis, some inter-current illnesses, and corticosteroids especially acute administration but less so with chronic administration. Interferon and cancer chemotherapy are also associated with CD4 decreases.<sup>8</sup> Sex, race, psychological stress, and physical stress have minimal effect on CD4. Pregnancy leads to hemodilution and a small decline in CD4 count, but no decline in CD4%.<sup>13</sup>

### **1.2.9 CD4 measurement**

CD4 is measured by either flow cytometric or non-flow cytometric methods. Flow cytometry is the gold standard for CD4 measurements and uses either Dual Platform (DP) or Single Platform (SP) techniques.<sup>8</sup> Non-Flow cytometric methods are either manual or non-manual. Manual methods include manual microscope based and Dynabead CD4 T lymphocyte quantitation.<sup>9</sup> Non manual methods include Microchip based and Semi-Bio manual slide technology methods.<sup>9</sup> In Swaziland, Ministry of Health accredited laboratories generally use the flow cytometric technique. WHO recommends that CD4 enumeration be done within 72hours from the time of vene-puncture. Generally, nearly all blood samples reach their final destination for analysis within 12hours with a few being run in the morning hours of the following day.

### **1.2.10 CD4 measurement: the problems**

Despite the importance of CD4 cell counts in HIV management significant inter-laboratory and intra-laboratory variations have been reported. There is widely published literature of CD4 enumeration problems and most are associated with laboratory analysis rather than biological differences. The

enumeration of blood CD41 (single-positive) T cells by flow cytometry is subject to errors such as counting CD41 monocytes or CD41cd81 (double-positive) T cells as CD41 T cells.<sup>14</sup> A recommendation by WHO is that both clinicians and patients must be aware of the variability in CD4 test results, especially if they will be used to make clinical decisions, such as initiation of antiretroviral therapy or opportunistic infection prophylaxis.<sup>15</sup> In another study, they cautioned against basing decisions on a single measurement.<sup>16</sup>

### **1.2.11 CD4 measurement: in-vitro previous studies**

There have been several studies related to CD4 count, clinical utility, accuracy and laboratory variability.

A study done in Malawi by MacLennan CA et al assessed diagnostic accuracy and clinical utility of a low cost CD4 count measurement method, Blantyre Count Assay (index tests). To assess accuracy, they measured agreement between the index test CD4 counts with two other reference methods, TruCount and FACS count, using a sample size of 119. To assess clinical utility, they compared CD4 counts from the index test and WHO clinical Staging in HIV positive patients. They found that the limits of agreement for Blantyre count and TruCount were excellent (cell count  $-48.9$  to  $27.0 \times 10^9/l$  for absolute counts in the CD4 range  $<400 \times 10^9/l$  and  $-2.42\%$  to  $2.37\%$  for CD4 percentage). For the clinical utility, of 193 patients with clinical stage I or II disease, who were ineligible for antiretroviral therapy by clinical staging criteria, 73 (38%) had CD4 counts  $<200 \times 10^9/l$ . By contrast, 12 (20%) of 60 patients with stage III or IV disease had CD4 counts  $>350 \times 10^9/l$ . They concluded that the index low cost test could be used interchangeably with the other comparator methods.<sup>17</sup>

In a similar study done in Ghana by Asong TN et al, they compared CD4 measurements between two laboratories using the FACS count system using a sample size of 11. They found that the inter-laboratory variability was significantly less with a Coefficient of Variation (CV %) of CD4 counts between the two laboratories of zero percent.<sup>18</sup>

Sax PE et al looked at the clinical implications of inter-laboratory variability in CD4 cell counts in patients with HIV infection. Using a sample size of twenty four, they found that fourteen (58.3%) had CD4 count results with enough variation to have led to conflicting treatment recommendations. They therefore concluded that when strict thresholds of CD4 cell counts are used as basis of treatment recommendation or for diagnosis of AIDS, inter-laboratory variability may be sufficient to alter the decisions made.<sup>16</sup>

Aboulker JP et al looked at the consistency of routine measurements of CD4 count, cd8 count and peripheral blood lymphocytes count. Their data showed that the consistency of CD4 measurements was satisfactory since the between-laboratory coefficient of variation for absolute CD4 cell numbers above  $200/mm^3$  was around 15% instead of 5–10% for all laboratories but one.<sup>19</sup>

Guarner J et al analyzed CD4+ T-lymphocyte variations in patients with advanced human immunodeficiency virus infection and counts below  $100 \text{ cells}/mm^3$  measured seven days apart. Using 55 patients, they collected samples between 08h00 and 10h00 seven days apart, and compared the two results. They found that the average total lymphocyte count on the first measurement was 1,064 (360-2,853), and on the second 1,162 (320-2,223;  $P = 0.07$ ); the percentage CD4 was 1.76 (0-8) on the first, and on the second 1.98 (0-9;  $P = 0.3$ ); the absolute CD4 cell count on

the first measurement was 16.6 (0-57) and on the second 22.8 (0-93;  $P = 0.01$ ). Statistically significant differences were found between the first and second absolute CD4 T-lymphocytes but not in the CD4 percentage. They thus concluded that for research protocols, repeating CD4+ cell determinations within a short period is advisable, to ensure a homogeneous population. On the other hand, for day-to-day patient follow-up, a combination of clinical criteria and both percentage and absolute CD4 cell counts should be used to make treatment decisions, because repeating CD4 cell measurements can be very costly.<sup>20</sup>

Jeganathan et al compared CD4 measurements by two flow cytometry methods, dual platform (DP) and single platform (SP) using 60 samples. DP flow cytometry yielded higher CD4 counts in 50/60 assays (83%). CD4 count and percentage by the two methods showed strong correlation for the counts ( $r=0.965$ ,  $P<0.0001$ ) and percentages ( $r=0.959$ ,  $P<0.0001$ ). Bland-Altman plot analysis showed that the limits of variation were within agreeable limits of  $\pm 2SD$  in 56/60 (93.3%) samples tested. Twenty-five (42%) samples had a difference of  $>50$  cells/mm<sup>3</sup>. Of these six (24%) exceeded 100 cells. They concluded that DP and SP methods had strong agreement.<sup>21</sup>

Edathodu J et al validated CD4 counts for the World Health Organization classification and clinical staging of HIV/AIDS. According to that validation, the correlation between the stages and the mean CD4 T-lymphocyte counts was -0.65. They concluded that the WHO clinical staging and classification of HIV/AIDS correlates well with CD4 T-lymphocyte counts.<sup>22</sup>

A study by Jimmerson et al concluded that CD4 percentages were more generalizable than absolute cell counts and hence CD4 percentages may be more reliable than absolute cell counts. CD4 lymphocyte counts were not generalizable across laboratories and thus not reliable for clinical decision making. In the same study they found that 14 percent of the variance across laboratories was associated with various sources of error rather than differences among patients.<sup>23</sup>

Thus various studies which have been done have shown conflicting results. In addition they used significantly different sample sizes and none of the articles explained sample size issues. For testing agreement, Bland and Altman (BA) recommended a sample size of 30 as an acceptable minimum but 50 as a "good" as it gives a 95% Confidence Interval (95% CI) about  $\pm 0.34s$ , where  $s$  is the standard deviation of the difference between measurements by the two methods.<sup>24</sup>

### **1.2.12 Possible sources of bias in CD4 measurement studies**

Selection bias, especially spectrum bias, may occur if study subjects do not include extreme values. Measurement bias, notably the Hawthorne effect, is possible especially if the laboratory technicians perform the tests in a much different way than is routinely done. Misclassification bias may be possible if results are mixed up through a clerical error.

Confounding bias is possible through the effects of biological factors such as diurnal or seasonal variations, pregnancy, and concomitant medications such as steroids or co-morbid diseases for example tuberculosis.

# CHAPTER: 2      STUDY MOTIVATION

## 2.1 MOTIVATION FOR THE STUDY

HIV/AIDS is a serious public health problem. Primary prevention measures are not as successful in Swaziland as hoped. Tertiary prevention of morbidity and mortality using HAART seems to be the cornerstone of mitigating the devastating effects of HIV/AIDS. HAART and CD4 cannot be separated.

Currently, CD4 counts for the Royal Swaziland Sugar Company (RSCC) hospital are being done at two different sites, the National Reference Laboratory (NRL) and the Good Shepherd Hospital (GSH). Generally the results take from one day to two weeks before they are available at HIV clinics although the time on results slip generally indicates that the tests are run between 12 and 24 hours. It would be valuable to investigate how the two results between NRL and GSH compare and what the impact on patient care is. At national level, some health centers do have low-cost flow cytometric CD4 machines but they are generally not in use. Clinicians and patients are highly suspicious of these low-cost machines. Patients opt to travel hundreds of kilometers to the NRL located at the national referral hospital.

Thus the motivation was to assess the presence of discrepancies, if any, in the RSCC CD4 collection practice, with emphasis on:

- ❖ The need to quantify the extent of intra and inter-laboratory variations if any. In addition, if the selected laboratories agree, can they be used interchangeably?
- ❖ The need for optimum care for HIV patients. In line with the WHO/UNAIDS global campaign of universal quality care, by ensuring that CD4 counts are reliable and repeatable, the RSCC would be giving patients quality care. Although not a primary motivation factor, this study provided an opportunity to perform a crude quality assurance check and accuracy of CD4 count results when compared with an accredited reference laboratory.
- ❖ The need to ensure that CD4 values are highly accurate, reliable and repeatable as this helps in decision making.
- ❖ There is a need to use limited resources (HAART) effectively. If CD4 results are not reliable, there is the potential of disease status misclassification. Are we really giving the correct interventions to those who really deserve them, in light of our limited resources effectively?
- ❖ From an ethical point of view, are we giving HAART to those who really deserve it and excluding those who do not qualify? This study will shed more light on how well the RSCC is distributing HAART. This will be directly an assessment of distributive justice from an ethical point of view. Indirectly this will be ensuring beneficence, by giving maximum benefit to those who need HAART, and also non-maleficence, by not exposing (initiating) patients who do not need HAART to potentially life threatening side effects of HAART treatment.
- ❖ Finally, there is need for further research on CD4 variability. This study may act as a prime step towards further studies on CD4 variability and possibly newer recommendations for initiating HAART which would not be solely dependent on CD4 only.

The above mentioned institutions', patients', ethical and circumstantial problems related to CD4 measurement, motivated this research.

## CHAPTER: 3      AIMS AND OBJECTIVES

### **3.1 Research Question**

How concordant and reliable are CD4 results performed at two different laboratories where samples are routinely sent from RSSC Hospital, in Swaziland: An analysis into the inter-laboratory and intra-laboratory variability of CD4.

### **3.2 Research Objectives**

The purpose of this study was to:

- i. To ascertain inter-laboratory agreement of CD4 counts between two routinely used laboratories the National Reference Laboratory (NRL) and the Good Shepherd hospital (GSH).
- ii. To quantify the extent of the inter-laboratory difference or agreement;
- iii. To determine the degree to which time lapse after sample collection contributes to the analytical CV.
- iv. To estimate the extent of disease misclassification due to CD4 variability below HAART initiation threshold
- v. To compare the degree of agreement of the RSSC Hospital CD4 results with an internationally accredited laboratory, Lancet.

# CHAPTER: 4

# METHODS

## 4.1 Study Design

The design was an analytical diagnostic, cross-sectional (observational) study.

## 4.2 Study Setting

The study was conducted at the HIV clinics of the Royal Swaziland Sugar Company (RSSC) hospital. The RSSC hospital, which comprises of two main sub-units, Simunye and Mhlume, is a sugar-cane estate based health facility located on the north-eastern part of Swaziland, in the Lubombo district, next to the borders with South Africa and Mozambique. It mostly offers primary health care to the residents of the estate and surrounding areas with a total catchment area of about 5000km<sup>2</sup>. The annual patient turn-over is in the region of 150 000patients per year.

Lubombo district is the worst hit by HIV in Swaziland. In response to the HIV pandemic, RSSC runs daily HIV clinics at each of the sub-units of Simunye and Mhlume. Due to financial constraints, the RSSC does not have an on-site CD4 cytometers and therefore send specimens to two laboratories, NRL and GSH on selected days. NRL is located about 200km away whilst GSH is only 70km away. NRL has introduced a free courier system to ensure the samples arrive on the day of vene-puncture. In addition, NRL ensures all samples are run on the same day.

Test samples from the study were sent to NRL and GSH as well as a third comparator laboratory, Lancet South Africa. Time was an important factor and therefore a special courier system provided by the principal investigator was used to ensure all samples arrived at set times.

## 4.3 Study Population and Sampling

### 4.3.1 Target population

- Confirmed HIV infected individuals
- Attended RSSC HIV/AIDS clinics during the study period for blood collection
- Due for CD4 either as baseline or follow up during study period.

### 4.3.2 Study Population

- Adults (>18years which is consenting age) patients regardless of WHO Stage
- Attended blood collection for CD4 during the study period
- Gave informed consent to the study.

### 4.3.3 Exclusion Criteria

- All patients attending HIV clinics , but not due for CD4 count (to avoid unnecessary risk of vene-puncture and its associated complications, in individuals who do not benefit from the results)
- Patients who did not consent to the whole process
- All patients below the age of consent (18years).

### 4.3.4 Sampling Method

A convenience sample representative of all WHO stages (Appendix 6) was used.

### 4.3.5 Sample Size

A sample size of fifty (50) was used. There is no precise statistical method for determination of sample size in studies of this nature. From the literature review, most previous studies used samples from ten to fifty, with only one major one using a hundred. Bland and Altman recommend a sample size of 30 as a “minimum acceptable” and 50 as “good” as it gives a 95% Confidence Interval about  $\pm 0.34s$ , where  $s$  is the standard deviation of the differences between measurements by the two methods.<sup>24</sup> Due to the snapshot nature of the methodology, issues of response rate were not influential.

### 4.3.6 Measurements

#### 4.3.6.1 Measurement tools

- Demographic data collection tool
- WHO Clinical Staging
- Standard amount of blood in EDTA tubes
- Time of collection to be documented
- Time when CD4 was performed was noted from the results slips or as per laboratory technician’s confirmed time
- Flow cytometric CD4 measurement methods at the respective laboratories

#### 4.3.6.2 Measurement methods

- Identified confirmed HIV positive patients coming for CD4 tests at the HIV clinics
- Staged patients using WHO Clinical Staging
- Selected convenience sample per each WHO category
- Got Informed Consent for collection of CD4
- Blood was collected and put into three EDTA tubes each for the three respective laboratories. Each of the samples was further split into two; one was to be done at or about 12hours from time of collection.
- The second sample was run approximately 12hours after the first or 24hours from time of vene-puncture.
- Entered demographic data, clinical stage, TB status, CD4 count and CD4% results obtained

### 4.3.7 Variables

- AGE (years): Age in years of participants.
- SEX: Sex of participants which can be Male/Female

- WEIGHT (kg): Weight of subjects in kilograms.
- WHO CLINICAL STAGE: Degree of immune-suppression: WHO Stage I; II; III; IV.
- OUT/IN-PATIENT: Whether patient was admitted or not
- HAART STATUS: On treatment/ Not on treatment
- PREGNANCY STATUS: Yes/No/Not applicable
- TUBERCULOSIS STATUS: Yes/No.
- TIME BLOOD COLLECTED: Clock at HIV clinic synchronized with laboratory clocks
- EXACT TIME CD4 RUN: Time was documented on the results sheet
- TIME DIFFERENCE: Time taken between collection and performing test
- CONCOMITANT DRUGS: The type of concomitant medications the patient is on.
- CD4 COUNT /CD4%: The results of CD4 counts/percentages

### **4.3.8 How bias was minimized**

To increase validity, bias was kept at a minimum in this study. Selection bias was avoided by including the full representative spectrum of patients from clinical stages one to four (Appendix 6). Confounding bias was not an issue because all the involved laboratories were equally affected by possible cofounders. Measurement bias, specifically the Hawthorne effect, was prevented by ensuring that the participating laboratory personnel were not alerted of the impending study. Disease misclassification was avoided by ensuring the three laboratories had physically distinct results slips.

### **4.3.9 Data Management and analysis**

#### ***4.3.9.1 Data Collection***

An electronic form was developed using Epidata with all the variables and limits. Similarly a manual form was developed for backup and for data cleaning purposes (see Appendix 1: Data Collection Tool).

#### ***4.3.9.2 Data Entry***

Data was entered twice into Epidata and normal protocols for data cleaning were used. Generation of new variables from the baseline data, for example logarithmic transformation of CD4, was developed where necessary.

#### ***4.3.9.3 Statistical Analysis***

Statistical analysis was done by the principal investigator with supervision by Professor Rheeder. The logical sequence, descriptive then analytical statistics was followed. Intra-laboratory repeatability of CD4 counts/percentages was analyzed by the Bland Altman (BA) method and calculating their coefficient of variation obtained from the two repeat specimens. Analysis for inter-laboratory variability was done using Bland Altman (BA) method. Assessment of the degree of misclassification of disease status was done using the 12hour sample from Lancet as the “truth”. These were compared with the 12hour results from GSH and NRL to assess the degree of misclassification.

# CHAPTER: 5

# RESULTS

## 5.1 Introduction

The results of the study were reported in the following sequence:

- a) Descriptive statistics
- b) Analytical statistics
- c) Discussion including limitations and conclusions drawn from the results

## 5.2 Descriptive Statistics

The results of descriptive statistics were reported as follows:

- General patient demographics
- Hypothesis testing for any inherent differences between the laboratories both at 12 and 24hours for CD4 count and CD4%.

### 5.2.1 Patient Demographics

Fifty three participants consented to participate in the study. Of these, 28 were males and 25 were females. The mean age of the participants was 37.4 years with a range of 19years to 57years. The mean weight was 64.8 kilograms. Six were on treatment for TB and no participant was pregnant since pregnant HIV positive women are attended at the Antenatal Clinic. 25 participants were on HAART.

The mean time to performing the first CD4 was 12hours. The mean time difference between the first and second CD4 was also 12hours for Lancet and NRL but 16hours 95% CI (14.9 to 17.01) for GSH. The mean CD4 for the Lancet laboratory was 366.7cells/mm<sup>3</sup> with 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentiles of 179 cells/mm<sup>3</sup>, 323 cells/mm<sup>3</sup> and 527.5cells/mm<sup>3</sup> respectively. For the NRL laboratory, the mean CD4 was 392.5cells/mm<sup>3</sup> with 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentiles of 184.3 cells/mm<sup>3</sup>, 353.3 cells/mm<sup>3</sup> and 547,5cells/mm<sup>3</sup>. Likewise for GSH, the values were 435.1cells/mm<sup>3</sup>, 240.5 cells/mm<sup>3</sup>, 403 cells/mm<sup>3</sup> and 615.5cells/mm<sup>3</sup> respectively. Details of demographic, clinical and laboratory characteristics are illustrated in Table 1 below.

**Table 1: Demographic, clinical and laboratory characteristics of participants**

<i>Demographic characteristics</i>				
Sex	<i>Males</i>	28	<i>Females</i>	25
Age:	37.4 (9.5)			
Weight:	64.8 (12.2)			
<i>Clinical characteristics</i>				
WHO Stage:	(i) 32.1 %	(ii) 22.6 %	(iii) 13.2%	(iv) 32.1%
On TB Treatment :	11.3%			
On HAART :	47.2%			

Pre-HAART :	52.8%
In-patients:	9.4%
Outpatients:	90.6%
Pregnant:	0%

**Laboratory parameters:**

Lancet	Observation (52)	Mean	25 <sup>th</sup> Centile	50 <sup>th</sup> Centile	75 <sup>th</sup> Centile
CD4 count at 12hours	52	373	181	336	539
CD4 count at 24hours	52	359	177	323	518
CD4 percentage at 12 hours	52	17	10	15	22
CD4 percentage at 24hours	52	17	10	15	21

NRL	Observation (52)	Mean	25 <sup>th</sup> Centile	50 <sup>th</sup> Centile	75 <sup>th</sup> Centile
CD4 count at 12hours	52	396	185	359	568
CD4 count at 24hours	52	389	183	346	535
CD4 percentage at 12 hours	52	18	11	17	24
CD4 percentage at 24hours	52	18	10	17	23

GSH	Observation (51)	Mean	25 <sup>th</sup> Centile	50 <sup>th</sup> Centile	75 <sup>th</sup> Centile
CD4 count at 12hours	51	439	249	397	611
CD4 count at 24hours	51	431	233	396	554
CD4 percentage at 12 hours	51	18	10	16	22
CD4 percentage at 24hours	51	18	10	16	22

**Timing of CD4 tests**

	Mean time in hours to running first CD4	Mean time in hours to running second CD4
Lancet	12.0	24.0
NRL	12.0	24.0
GSH	11.6	24.7

## 5.2.2 Assessment of CD4 mean differences across laboratories

From Table 1 above, the mean of the CD4s from the three laboratories appeared different. Could this difference be due to some inherent variations in the random sample means or was the difference due to the fact that the measurement of samples from the laboratories was indeed different?

To assess if there indeed was a difference, hypothesis testing was done using a paired t-test. No adjustment for multiple testing was done. The p-values for between laboratories 12 hour CD4 comparisons were as follows: Lancet/NRL 0.02, Lancet/GSH 0.00 and GSH/NRL 0.05. At 24hours the p-values were 0.01, 0.00 and 0.03 for the laboratory combinations above respectively. For the 12hour CD4% the p-values were 0.11, 0.00 and 0.56 whilst at 24hours p-values were 0.09, 0.00 and 0.91 for the three laboratory combinations respectively.

## 5.3 ANALYTICAL STATISTICS

### 5.3.1 Introduction

Results for analytical statistics were discussed in three distinct sections:

- i. Intra-laboratory variability analysis which analysed the degree of repeatability of CD4 count, CD4 percent (CD4%) and where indicated, percentage difference of CD4 (% difference of CD4 count) for each of the three laboratories using the 12 and 24hours tests results.
- ii. Inter-laboratory variability compared CD4 counts and CD4% (where indicated percentage difference of CD4 count) between the three laboratories, that is Lancet/NRL, Lancet/GSH and GSH/NRL, at 12hours and 24hours
- iii. The final stage reported the degree of disease misclassification

### 5.3.2 Intra-laboratory variability

The results for intra-laboratory variability were reported in the following sequence:

- a. The coefficient of variability% (CV%) of absolute CD4 count and of CD4% compared the 12 hour and 24hour test results for each of the three laboratories.
- b. Bland and Altman (BA) analysis of the CD4, CD4% and %difference of CD4 count. The 12hour and 24hours results were compared for agreement. The BA analysis composed of calculating bias and limits of agreement, with corresponding 95% CI for each of CD4, CD4% and %difference of CD4 count with BA plots analysis.

#### ***5.3.2.1 Coefficient of variability % (CV%) for absolute CD4 count and CD4%***

The coefficient of variability percentage (CV%) compared the 12 and 24hour CD4 counts and CD4%, for each of the three laboratories, Lancet, NRL and GSH. The CV% of the absolute CD4 count between the first and second tests was 3.5%, 8.4% and 20.1% for NRL, Lancet and GSH respectively. For the corresponding CD4 percent (CD4%), the CV% was 8.34%, 5.6%, 7.5% for NRL, Lancet and GSH respectively.

#### ***5.3.2.2 Bland and Altman (BA) analysis***

##### **a) CD4 absolute count and CD4%**

The Bland and Altman method was subsequently used to evaluate the repeatability of CD4 count, CD4% and %difference of CD4 count. The biases and limits of agreement were as per Table 2 below. The bias was for CD4% was 0.1 (95% CI -0.2 to 0.3) for Lancet, -0.3 (95% CI -0.7 to 0.1) and 0.1 (95% CI -0.3 to 0.5). Appendices 8 and 9 shows the corresponding BA plots for CD4 count and CD4% for the intra-laboratory variability for Lancet, GSH and NRL respectively.

**Table 2: Intra-laboratory bias and limits of agreement for CD4, CD4% and %difference in CD4 between 12 and 24hours tests**

	Bias( 95% CI)	LIMITS OF AGREEMENT	
		Lower (95% CI)	Upper (95% CI)
<i>Absolute CD4 Count</i>			
Lancet	13.5 (5.0 to 21.9)	-46.0 (-60.6 to -31.5)	73.0 (58.5 to 87.6)
GSH	8.2 (-16.0 to 32.4)	-160.5 (-202.2 to -118.9)	176.9 (135.3 to 218.6)
NRL	7.0 (3.2 to 10.7)	-19.5 (-25.9 to -13.0)	33.4 (26.9 to 39.9)
<i>CD4%</i>			
Lancet	0.1 (-0.2 to 0.3)	-1.7 (-2.2 to -1.3)	1.9 (1.5 to 2.4)
GSH	-0.3 (-0.7 to 0.1)	-2.9 (-3.5 to -2.2)	2.3 (1.7 to 3.0)
NRL	0.1 (-0.3 to 0.5)	-2.8 (-3.5 to -2.1)	3.0 (2.3 to 3.7)
<i>%difference of CD4</i>			
Lancet	3.6 (0.35 to 6.9)	-19.4 (-25.0 to -13.8)	26.6 (21.0 to 32.2)
GSH	-0.45 (-6.3 to 5.4)	-41.3 (-51.4 to -31.2)	40.4 (30.3 to 50.5)
NRL	2.8 (1.2 to 4.5)	-8.5 (-11.3 to -5.8)	14.2 (11.4 to 17.0)

### b) Variability of percentage difference of CD4 (%difference of CD4)

Lancet had a bias of 3.6 (95% CI 0.35 to 6.9) and for NRL it was 2.8 (95% CI 1.2 to 4.5). The bias for GSH was -0.45 but the 95% CI include zero, -6.3 to 5.4. The corresponding BA plots for the CD4 percentage differences are shown in Appendix 10.

## 5.3.3 Inter-laboratory variability

Bland and Altman analysis, Concordance and Pearson correlation coefficients were used to assess between laboratory agreements. The results were reported as follows:

a) Inter-laboratory variability at 12hours comparing Lancet/NRL, Lancet/GSH and GSH/NRL and specifically analyzing;

- i) Absolute CD4 count and CD4%
- ii) %difference of CD4 count

b) Inter-laboratory variability at 24hours comparing Lancet/NRL, Lancet/GSH and GSH/NRL and specifically analyzing;

- i) Absolute CD4 count and CD4%
- ii) %difference of CD4 count

c) Concordance correlation coefficient, Pearson correlation coefficient and the corresponding strength of agreement

d) Disease misclassification

BA analysis was the main analytical method, the steps in which the results are reported was as follows:

- Calculation of mean difference/bias and limits of agreements with the respective 95% confidence intervals
- Assessment of relationship pattern between CD4 difference and average using BA plots .
- To add value, a %difference was also plotted

### 5.3.3.1 Inter-laboratory variability at 12hours

#### a) Variability of absolute CD4 count and CD4% at 12hours

From the BA analysis, the mean bias for Lancet/NRL for 12hour CD4 count was -31.5 (95% CI -57.6 to -5.5) with wide limits of agreement between -213.3 (95% CI -258.2 to -168.4) to 150.2 (95% CI 105.3 to 195.1). For Lancet /GSH bias was -64.3 (95% CI -81.6 to -47.0) with fairly narrow limits of agreement, a lower of -183.8 (95% CI -213.6 to -154.0) and an upper of 55.2 (95% CI 25.4 to 85.0). GSH/NRL had limits of agreement between -300.2 (lower) and 223.9 (upper) and a low bias, -38.2(95%CI -75.6 to -0.6). Table 3 summarises CD4 count, CD4% and %difference of CD4 count at 12hours post vene-puncture. The corresponding BA plots are shown in Appendices 11, 12 and 13.

For CD4% at 12hours the Lancet/GSH bias was -0.7 (95% CI -1.1 to -0.4) with lower limits of -3.1 (95% CI -3.7 to -2.5) and upper limits of 1.7 (1.1 to 2.2). For Lancet/NRL and GSH/NRL, the mean difference was -1.2 (95% CI -2.7 to 0.3) and 0.5 (95% CI -1.1 to 2.1) respectively and of note the 95% CIs for both included zero.

**Table 3: Inter-laboratory bias and limits of agreement for CD4 count, CD4% and %difference CD4 at 12hours**

	Bias ( 95% CI)	LIMITS OF AGREEMENT	
		Lower (95% CI)	Upper (95% CI)
<i>CD4 Count at 12hours</i>			
Lancet/NRL	-31.5 (-57.6 to -5.5)	-213.3 (-258.2 to -168.4)	150.2(105.3 to 195.1)
Lancet/GSH	-64.3 (-81.6 to -47.0)	-183.8 (-213.6 to -154.0)	55.2 (25.4 to 85.0)
NRL /GSH	-38.2 (-75.6 to -0.6)	-300.2 (-364.8 to -235.5)	223.9 (159.2 to 288.5)
<i>CD4% at 12hours</i>			
Lancet /NRL	-1.2 (-2.7 to 0.3)	-11.7 (-14.3 to -9.1)	9.3 (6.7 to 11.9)
Lancet/GSH	-0.7 (-1.1 to -0.4)	-3.1 (-3.7 to -2.5)	1.7 (1.1 to 2.2)
NRL /GSH	0.5 (-1.1 to 2.1)	-10.7 (-13.4 to -7.9)	11.6 (8.9 to 14.4))
<i>% difference of CD4</i>			
Lancet /NRL	-8.9 (-19.1 to 1.4)	-80.4 (-98.1 to -62.7)	62.7 (45.0 to 80.4)
Lancet/GSH	-19.2 (-23.2 to -15.1)	-47.1 (-54.0 to -40.1)	8.8 (1.8 to 15.7)
NRL /GSH	-11.2 (-23.4 to 1.0)	-96.2 (-117.2 to -75.2)	73.9 (52.9 to 94.9)

#### b) Variability percentage %difference CD4 count at 12hours

The %difference of CD4 count for Lancet/GSH had a bias of -19.2 (95% CI -23.2 to -15.1) and a corresponding narrow limits of agreement, -47.1 (95%CI -54.0 to -40.1) to 8.8 (95% CI 1.8 to 15.7), as per Table 3 above.

### 5.3.3.2 Inter-laboratory variability: 24 hours

#### a) Variability of CD4 count and CD4% at 24hours

At 24hours the results of CD4 count variability were similar to the 12hours trend for Lancet/NRL and NRL/GSH. For Lancet/GSH the bias was 8.2 (-16.0 to 32.4). Detailed summary for CD4 count, CD4% and %difference of CD4 was as per table 4 and the corresponding BA plots are in the Appendices 14, 15 and 16.

At 24hours, the bias for Lancet/GSH was -1.1 (-1.6 to -0.5) and limits of agreement, lower 4.9 (95% CI -5.8 to -3.9) and upper 2.7 (95% CI 1.8 to 3.6). The Lancet/NRL or NRL/GSH mean differences were not statistically significant, -1.2 (95% CI -2.5 to 0.2) and 0.1 (95% CI -1.3 to 1.5), respectively.

### b) Variability of %CD4 difference

At 24hours, only the NRL and GSH could be used interchangeably based on the bias of -14.2 (95% CI -27.2 to -1.1) and narrow limits of agreement, lower of -105.1 (95% CI -127.5 to -82.6) and upper of 76.7 (95% CI 54.2 to 99.2). The biases for Lancet/GSH and Lancet NRL included zero, no difference. Appendix 16 shows the trends on BA plots.

**Table 4: Inter-laboratory bias and limits of agreement for CD4 count, CD4% and %difference of CD4 at 24hours**

	Bias( 95% CI)	LIMITS OF AGREEMENT	
		Lower (95% CI)	Upper (95% CI)
<i>CD4 Count at 24hours</i>			
Lancet/NRL	-35.6 (-60.0 to -11.1)	-205.7 (-247.6 to -163.7)	134.5 (92.5 to 176.5)
Lancet/GSH	8.2 (-16.0 to 32.4)	-195.0 (-227.6 to -162.5)	65.8 (33.3 to 98.3)
NRL /GSH	7.0 (3.2 to 10.7)	-265.0 (-321.3 to -208.7)	191.4 (135.0 to 247.7)
<i>CD4% at 24hours</i>			
Lancet /NRL	-1.2 (-2.5 to 0.2)	-10.5 (-12.8 to -8.2)	8.2 (5.7 to 10.5)
Lancet/GSH	-1.1 (-1.6 to -0.5)	-4.9 (-5.8 to -3.9)	2.7 (1.8 to 3.6)
NRL /GSH	0.1 (-1.3 to 1.5)	-9.7 (-12.1 to -7.3)	9.9 (7.4 to 12.3)
<i>% difference of CD4</i>			
Lancet /NRL	-8.6 (-18.5 to 1.3)	-77.6 (-94.6 to -60.5)	60.4 (43.4 to 77.5)
Lancet/GSH	-21.7 (-28.3 to 15.1)	-67.0 (-78.3 to -55.7)	23.6 (12.3 to 34.9)
NRL /GSH	-14.2 (-27.2 to -1.1)	-105.1 (-127.5 to -82.6)	76.7 (54.2 to 99.2)

### 5.3.4 Inter-laboratory variability: Pearson and Concordance Correlation Coefficient

The concordance correlation coefficient for absolute CD4 count at 12hours was 0.95 for Lancet /GSH, corresponding to strength of agreement graded as substantial. For Lancet/NRL both the Pearson and concordance correlation were 0.93 whilst for NRL/GSH both showed poor strength of agreement, as per Table 5 below.

The CD4% for Lancet/GSH had an almost perfect agreement at 12hours. For Lancet/NRL and NRL/GSH the CD4% at 12hours had a poor strength of agreement. At 24hours, the CD4 count for Lancet/NRL and NRL/GSH had moderate and poor strength of agreement, respectively. Lancet/GSH lost its substantial agreement to become moderate. A similar trend was followed with the CD4%.

**Table 5: Correlation of CD4 count and CD4% at 12 and 24hours**

	Concordance correlation Coefficient	Strength of Agreement	Pearson Coefficient
<i>CD4 Count at 12hours</i>			
Lancet/NRL	0.93 (0.88 to 0.96)	moderate	0.93
Lancet/GSH	0.95 (0.92 to 0.97)	substantial	0.98
NRL /GSH	0.88 (0.79 to 0.93)	poor	0.89
<i>CD4% at 12hours</i>			
Lancet /NRL	0.85 (0.76 to 0.91)	poor	0.86
Lancet/GSH	0.99 (0.98 to 0.99)	almost perfect	0.99
NRL /GSH	0.84(0.74 to 0.91)	poor	0.85
<i>CD4 count at 24hours</i>			
Lancet/NRL	0.94 (0.89 to 0.96)	moderate	0.95
Lancet/GSH	0.94( 0.90 to 0.96)	moderate	0.97
GSH/NRL	0.90 ( 0.83 to 0.94)	poor	0.91
<i>CD4 % at 24hours</i>			
Lancet /NRL	0.88 (0.80 to 0.93)	poor	0.89
Lancet/GSH	0.97 (0.95 to 0.99)	substantial	0.98
NRL /GSH	0.88 (0.80 to 0.93)	poor	0.88

### 5.3.5 Disease Misclassification: Impact of relying on single CD4 on HAART initiation

Finally, an inter-rater agreement was performed to assess the degree of agreement at HAART initiation threshold. The agreement was 81.1% for Lancet/NRL, 88.7% for Lancet/GSH and 77.4% for NRL/GSH corresponding to Kappa values of 0.64 which corresponds to good agreement using Byrt's criteria, (see Appendix 7), 0.77 (good agreement) and 0.55 (fair agreement) respectively as shown in Table 6 below.

**Table 6: Disease misclassification at HAART initiation threshold**

Lab	Agreement	Expected Agreement	Kappa	Standard Error	z	Prob > z
Lancet/NRL	81.1%	48.4%	0.64	0.13	4.96	0.00
Lancet/GSH	88.7%	49.9%	0.77	0.14	5.70	0.00
NRL/GSH	77.4%	50.23	0.55	0.13	4.07	0.00

### 5.3.6 Comparison of categories for HAART initiation: McNemar test for paired proportions

Finally, a test of marginal homogeneity was done using a comparison for the paired proportions for those who required HAART was performed using McNemar tests and the results were as per table 7 below.

**Table 7: Comparison of paired proportions for HAART initiation categories using McNemar test**

Lab	McNemar's value	z	P value	Difference	95% CI of difference
Lancet/NRL	3.11	3.84	0.05	0.18	-0.017 to 0.33
Lancet/GSH	1.57	3.84	0.14	1.25	-0.060 to 0.28
NRL/GSH	0.64	3.84	0.47	0.09	-0.012 to 0.28

The McNemar values were 3.11, 1.576 and 0.64 for Lancet/NRL, Lancet/GSH and NRL/GSH respectively, and were all less than the critical value, 3.84. In addition, the 95% CI of the differences included zero for all the three cases. We therefore fail to reject the null hypothesis of no difference and conclude that the proportions were the same.

## CHAPTER: 6

## DISCUSSION

### *Introduction*

In Swaziland, and most Southern African countries, CD4s are done at central stations. Samples are usually collected from satellite clinics by various transport modes and sent to the central stations. In Swaziland, there are several satellite stations at district level. The time it takes to reach the satellite station varies but generally speaking all specimens reach their final destination within 12hours. Most laboratories run their first batch of samples on the evening using an on-call laboratory technologist. Some specimens often spill-over and are run on the following morning.

### *Comparison of the participating laboratories*

The participating laboratories had different CD4 count and CD4 percentage means both at 12 and 24hours. One obvious question was whether the differences were due to inherent variations or merely by chance. To rectify the cause of the difference, a paired t-test was performed for the CD4 count and CD4% between the participating laboratories: Lancet/NRL, Lancet/GSH and GSH/NRL. The p-values for between laboratories 12 hour CD4 comparisons were as follows: Lancet/NRL 0.02, Lancet/GSH 0.00 and GSH/NRL 0.05. At 24hours the p-values were 0.01, 0.00 and 0.03 for the laboratory combinations above respectively. The p-values for the laboratories compared were less than 0.05 at 12 and 24hours, thus implying existence of inherent differences in CD4 counts between the participating laboratories.

For the 12hour CD4% the p-values were 0.11, 0.00 and 0.56 whilst at 24hours p-values were 0.09, 0.00 and 0.91 for the three laboratory combinations respectively. Thus, there were no inherent differences of CD4% between Lancet/NRL and GSH/NRL both at 12 and 24hours based on p-values greater than 0.05, implying we fail to reject the null hypothesis,  $H_0$ . On the contrary, there were indeed some differences between Lancet and GSH CD4% both at 12 and 24hours.

### *Intra-laboratory variability*

The CV% for CD4 absolute count was 3.5%, 8.4% and 20.1% for NRL, Lancet and GSH respectively, implying a good repeatability for NRL and Lancet. GSH had poor repeatability of CD4 count, the CV% being 20.1%. For CD4% the CV% was even stronger: 8.3%, 5.6% and 7.5% for NRL, Lancet and GSH respectively. Thus the CD4% percentage had good repeatability for all the three laboratories although weaker for NRL when compared to the repeatability of CD4 count: 3.5% versus 8.3%. Because CV% is generally regarded as a weaker statistical tool for repeatability measurement, it was necessary to perform BA.

The bias for Lancet was 13.5 (95% CI 5.0 to 21.9) with narrow limits of agreement, the lower being -46.0 (-60.6 to -31.5) and the upper 73.0 (58.5 to 87.6). Thus for a given CD4 at 12hours, the 24hour reading could be 46cell/mm<sup>3</sup> less or 73cell/mm<sup>3</sup> higher. This difference was small enough not to impair clinical decision. For NRL, the bias was closer to zero than for Lancet, 7.0 (95% CI 3.2 to 10.7) and the limits of agreement were narrower, lower being -19.5 (95% CI -25.9 to -13.0) and upper 33.4 (26.9 to 39.9), a good example of perfect repeatability. The narrow limits of agreement would not affect clinical decision making and can therefore be safely interchanged. The bias for GSH was 8.2

(95% CI -16.0 to 32.4) and limits of agreement of -160.5 (95% CI -202.2 to -118.9), for lower and 176.9 (95% CI 135.3 to 218.6) for upper limit. Thus the 95%CI included zero, a statistically significant result, which implies no difference and therefore high repeatability. But if one looks at the very wide limits of agreement, the statistical significance does not translate to clinical significance-the beauty of BA analysis!

For CD4%, the biases were 0.1 (95% CI -0.2 to 0.3), -0.3 (95% CI -0.7 to 0.1) and 0.1 (-0.3 to 0.5) for Lancet, GSH and NRL respectively. Thus for the three laboratories, the bias had a 95% CI which included zero, no difference. Statistically, the 12hour and the 24hour results were the same. For clinical decision making, the limits of agreement would be necessary. In this case, the limits were narrow for all the three laboratories: -1.7 to 1.9; -2.9 to 2.3; -2.0 to 3.0 for Lancet, GSH and NRL respectively. Surely, these narrow limits would not interfere with clinical decision making and we therefore conclude that the CD4% was highly repeatable statistically and clinically.

To add value and to compensate for the BA plot dispersion as the mean CD4 increased, percentage CD4 difference (%CD4 difference) analysis was performed. The bias for GSH was -0.45 (95% CI -6.3 to 5.4) which implied no difference between 12hour and 24hour samples. The bias was 3.6 (95% CI 0.35 to 6.9) for Lancet and 2.8 (1.2 to 4.5) for NRL which were close to zero and hence confirms repeatability. The corresponding limits of agreement were narrow not to impair clinical decision making.

**Table 8: Summary for intra-laboratory variability**

Laboratory	Statistical agreement	Clinical interchangeable
<i>CD4 COUNT</i>		
Lancet	yes	yes
GSH	yes	no
NRL	yes	yes
<i>CD4%</i>		
Lancet	yes	yes
GSH	yes	yes
NRL	yes	yes

#### *Inter-laboratory variability at 12hours*

For assessing agreement, relying on bias alone without reference to the limits of agreement and 95% CI can be very misleading as was seen in this study. The biases for CD4 absolute count at 12hours for Lancet/NRL and NRL/GSH had some good precision based on the narrow 95% CI: -31.5 (95% CI -57.6 to -5.5) and -38.2 (95% CI -75.6 to -0.6) respectively. Thus for every Lancet CD4 count, NRL would have a higher reading by a mean of 31.5cells/mm<sup>3</sup> whilst for every NRL reading, GSH would have a higher reading by 38.2cells/mm<sup>3</sup>. The biases were both close to zero. Surely, a mean difference of 31.5 and 38.5 would not affect clinical decision making? A moment of looking at the limits of agreement could lead to changing decision: -213.3 to 150.2 for Lancet/NRL and -300.2 to 223.9 for NRL/GSH. This implies for a particular NRL CD4 count, Lancet would have any value between 213.2 cells/mm<sup>3</sup> less or 150.2 cells/mm<sup>3</sup> higher; GSH would similarly have a corresponding value 300.2 cells/mm<sup>3</sup> less or 223.9 cells/mm<sup>3</sup> higher. That would be unacceptable in clinical practice if the two were to be interchanged. In contrast, bias for Lancet/GSH was a precise -64.3 (95% CI -81.6 to -47.0), which was a bit further from zero compared to the Lancet/NRL and NRL/GSH. The limits of agreement were more precise and narrower, -183.8 to 55.2. An arbitrary patient with a CD4 of 200cells/mm<sup>3</sup> from GSH would have CD4 of between 16.2 and 255.2cells/mm<sup>3</sup>. The limits are narrow

enough not to impair clinical judgment Percentage difference of CD4 plots, a mechanism to counter increased dispersion of bias as mean CD4 increased, confirmed similar results. Table 8 below summarises the statistical and clinical implications of these results.

Besides having an excellent intra-laboratory agreement, CD4% maintained the agreement across the laboratories at 12 hours. The inter-laboratory biases were as follows: Lancet/NRL -1.2 (95% CI -2.7 to 0.3); Lancet/GSH -0.7 (95% CI -1.1 to -0.4); NRL/GSH 0.5 (95% CI -1.1 to 2.1). Thus there was no difference between Lancet/NRL and NRL/GSH because the 95% CI included zero for both cases. For Lancet/GSH, the bias was very close to zero and highly precise. The limits of agreement were -11.7 to 9.3 for Lancet/NRL; -3.1 to 1.7 for Lancet/GSH; -10.7 to 11.6 for NRL/GSH. The range for limits of agreement of +20% would affect clinical decision making. Hence Lancet/NRL and NRL/GSH would not be interchangeable. As for the CD4 count, only Lancet/GSH CD4% results may be used interchangeably in clinical practice.

#### *Inter-laboratory agreement at 24 hours*

At 24 hours, the CD4 count maintained similar trends to 12 hours, possibly a sign of CD4 stability both within (see intra-laboratory variability discussion above) and across laboratories. For Lancet/GSH the bias was -64.6 (95% CI -83.5 to -45.7) [compare with -64.3 (95% CI -81.6 to -47.0) at 12 hours] and limits of agreement of, lower -195.0 (95% -227.6 to -162.5) to, upper 65.8 (95% CI 33.3 to 98.3). Thus the degree of agreement between Lancet/GSH was not affected much by the time difference of performing the test. The Lancet/NRL and NRL/GSH results also maintained 12 hour trends and were both not interchangeable based on the wide limits of agreement.

The CD4% bias had a 95% CI which included zero for all the three laboratory combinations and therefore were not different. For Lancet/NRL the bias was -1.2 (95% CI -2.5 to 0.2) limits of agreement -10.5 to 8.2; Lancet/GSH: -1.1 (95% CI -1.6 to 0.5); limits of agreement -4.9 to 2.7; NRL/GSH 0.1 (95% CI -1.3 to 1.5) limits of agreement -9.7 to 9.9.

Another complementary statistical tool, concordance correlation coefficient echoed the obtained using BA analysis confirming a strong agreement between NRL/GSH combination. The Pearson's correlation, a tool for measuring association, showed strong association.

**Table 9: Summary for inter-laboratory variability of CD4 count at 12 hours**

CD4 count at 12 hours	Statistical Agreement	Clinically Inter-changeable
Lancet/NRL	Yes	No
Lancet/GSH	Yes	Yes
GSH/NRL	Yes	No

**Table 10: Summary for inter-laboratory variability of CD4% at 12 hours**

CD4 percent (CD4%) at 12 hours	Statistical Agreement	Clinically Inter-changeable
Lancet/NRL	yes	No
Lancet/GSH	Yes	Yes
GSH/NRL	yes	No

**Table 11: Summary for inter-laboratory variability of CD4 count at 24hours**

CD4 percent (CD4%) at 24hours	Statistical Agreement	Clinically Inter-changeable
Lancet/NRL	yes	No
Lancet/GSH	Yes	Yes
GSH/NRL	yes	No

**Table 12: Summary for inter-laboratory variability of CD4% at 24hours**

CD4 percent (CD4%) at 24hours	Statistical Agreement	Clinically Inter-changeable
Lancet/NRL	yes	No
Lancet/GSH	Yes	Yes
GSH/NRL	yes	No

### *Disease misclassification*

Finally, an inter rater-agreement was performed at the HAART initiation threshold, 350cells/mm<sup>3</sup>. The agreement was 81.1% for Lancet/NRL, 88.7% for Lancet/GSH and 77.4% for NRL/GSH corresponding to Kappa values of 0.64, 0.77 and 0.55 respectively. Thus below the HAART initiation threshold, GSH may miss about 11.3% of patients compared to 18.9% for NRL when compared with the international comparator laboratory, Lancet. Between themselves, NRL and GSH would potentially miss up to 22.6% of patients who would qualify for HAART if they interchange their tests.

The null hypothesis of the two proportions having the being the same was not rejected in all the three cases since the calculated McNemar values were all less than the critical value, 3.84, the p-values were higher level of significance and the 95% CI involved zero for all the three permutations. The proportions of patients who qualified for HAART were thus not different for Lancet/GSH, Lancet/NRL and NRL/GSH.

### *What is already known on this topic?*

- CD4 is the key investigation before HAART initiation, especially in line with the latest treatment guidelines.
- There is some inherent biological and laboratory variation of CD4 count during enumeration.
- Generally, CD4 enumerating machines use the same technique, flow cytometry, and tend to agree.
- CD4 remains stable within 72hours after vene-puncture and therefore WHO recommend CD4s must be done within 72hours from vene-puncture.
- In Swaziland, most CD4 tests are done within 12 to 24hours post vene-puncture.
- No similar studies have been done in Swaziland and the region in general.

### *What this study adds*

- The laboratories used in this study in Swaziland have good intra-laboratory variability within 12 and 24hours.
- Despite good intra-laboratory variability and using the same measurement technique, flow cytometry, there is significant variation across our two main laboratories, namely NRL and GSH.

- CD4% has excellent precision within and across laboratories and future studies should try to validate criteria which uses CD4% to make clinical decisions.
- RSSC can use Lancet and GSH laboratories interchangeably for CD4 count and/or CD4%
- There is good statistical agreement (but not clinical) of CD4 count and CD4% between NRL and GSH laboratories regardless of whether the samples are run at 12 hours or 24 hours
- Neither CD4 count nor CD4% can be confidently interchanged between NRL and GSH without affecting clinical decision making.
- GSH CD4% has perfect agreement, hence interchangeable, with an accredited international laboratory, Lancet, in this study.
- CD4 count was fairly stable over 12 and 24 hours
- Both GSH and NRL have some statistically significant agreement with a reputable international laboratory but this is not clinically useful as the limits of agreement are too wide.
- Despite poor agreement with a comparator laboratory, NRL is more sensitive in identifying patients who need to be initiated on HAART than GSH.
- Future studies should strive to eliminate inherent differences between laboratories. The differences, generally analytical/laboratory variations, could have been due to equipment calibration which was not validated before commencing the study, technical skills/competences of the laboratory technicians, differences in brands of the equipment and clerical mistakes associated with processing the samples from reception area to the final destination, the flow cytometric machines or the transportation of samples.

### *Conclusion*

- CD4% remains stable between 12 and 24 hours post blood collection
- Lancet, GSH and NRL have good statistical repeatability of CD4 count between 12 and 24 hours. However only Lancet and NRL and clinical interchangeability.
- Lancet and GSH CD4 count and CD4% can be used interchangeably without affecting clinical decision making.
- NRL and GSH, the two major national laboratories in Swaziland in the study, cannot be used interchangeably without affecting clinical decision making.
- National HIV programs should standardize standards and ensure stringent Standard Operating Procedures (SOPs) between laboratories in the country
- Our laboratories need to regularly validate their CD4 machines in keeping with international comparator laboratories standards.
- The national guidelines should insist on two CD4 results before making critical decisions such as initiating HAART, to systematically reduce error. Clinicians should continue to use their clinical acumen in decision making and be vigilant when they get suspicious results.
- 11% of patients who qualify for HAART are missed by GSH and 23% by NRL if CD4 alone is used as the initiation criterion. Continuous quality improvement cycles need to be initiated to improve on the high disease misclassification.
- The proportion of patients affected by these inter and intra laboratory variations were similar when all the three laboratories were compared.
- National programs in Sub-Saharan Africa, being the worst affected and economical disadvantaged, should seriously assess the economic and ethical implications of CD4 variability to reduce morbidity, mortality and economic cost

*Strength of this study*

- By being pragmatic, this study tried to analyze the current standard of practice of enumerating CD4 for a national program as opposed to validating laboratory techniques.
- In addition, it assessed the impact of time delays when performing CD4 tests.

*Weaknesses of this study*

- Clinically meaningful limits of agreement should have been decided *a priori*.
- The study only looked at the laboratory process of CD4 measurement and ensured that possible logistics problems like sample mix-up were kept to a minimum and thus was not as pragmatic as possible.
- There were inherent differences for CD4 count both at 12 and 24hours after pairing the results, the cause of which we failed to account for.
- The sample size was just “good” enough due to financial costs. It would have been ideal to use a sample size of at least 100.
- CD4 samples for Lancet laboratory were sent to South Africa by a freight vehicle on a daily basis. We do not know the implications of transportation conditions on the eventual CD4 count/CD4%.

## CHAPTER: 7

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# CHAPTER: 8

# APPENDICES

## Appendix 1: Data Collection Tool

### Demographics

ID Number		ART Number	
Date of Birth		Sex	

### Clinical Parameters

Weight in Kilograms	.....Kilograms	Height in meters	..... Meters
Pregnant	Yes / No	Current Tuberculosis	Yes / No
WHO Stage	1 / 2 / 3 / 4	On HAART	Yes / No
Other medications	Yes / No	Admission Status	In patient/ Out patient

### Laboratory Parameters

	Lancet Test	GSH Lab Sample 1	GSH Lab Sample 2	NRL Lab Sample 1	NRL Lab Sample 2
Date Collected					
Time Collected					
Date CD4 done					
Time CD4 done					
CD4 Results					

## **Appendix 2: Ethics and Legal Considerations**

### **Approval of study by the relevant departments**

The study was submitted to the AAC at the SHSPH and approved as Protocol 74/2010 on 21<sup>st</sup> of April 2010.

### **Informed Consent**

Informed consent was sought from the patient. Document on the full procedure of vene-puncture including possible adverse consequences was developed, see Appendix XXXX.

### **Privacy of Information / Confidentiality**

Strict confidentiality was maintained for all patients in line with Swaziland Health Professions Council HIV Confidentiality clauses. The results will be kept at SHSPH for the next 25 years.

### **Potential Harms and Benefits**

As expected, this did not have any harm on any of the participants, for example, hematoma formation, pain or hemorrhage. Samples were collected only from patients who had come for CD4 collection.

### **Justice**

Legal justice issue-Consultation was made with the laboratory or hospital heads of all participating laboratories to alert them of the intended study.

### **Conflict Of Interest**

The Principal Investigator, Dr Mlawanda does not have affiliations with any of the laboratories involved in this research. In addition to using this dissertation for my Masters in Clinical Epidemiology with the University of Pretoria, it will also be published in a Peer Review Journal and presented at one of the HIV clinicians' congress so it can pave the way to more studies of a similar nature.

### **Appendix 3: Patient Information Leaflet & Informed Consent**

TITLE OF STUDY:

Inter-laboratory and Intra-laboratory variability of CD4 counts: A pragmatic Analysis

Dear Mr. / Mrs. .... date ...../...../.....

#### **1) INTRODUCTION**

You are invited to volunteer for a research study. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. In the best interests of your health, it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study, wherever possible.

#### **2) THE NATURE AND PURPOSE OF THIS STUDY**

You are invited to take part in a research study. First, I understand you came here today to do your CD4 counts, which is a test that determines how badly injured one's health is due to HIV. The aim of this study is to evaluate the reliability of CD4 counts done in our laboratories. By doing so we wish to learn more about how trustworthy are our results of CD4 and ensure that we are giving you the best of care. Some problems could be serious and if identified early could save you from having problems later on. Thus we do not want to give you treatments too early or too late based on inaccurate results.

#### **3) EXPLANATION OF PROCEDURES TO BE FOLLOWED**

This study involves answering some questions with regard to your illness, examination of yourself, your general health, weight and height and whether you have been treated for Tuberculosis (TB). Vitals, which refer to temperature, pulse and blood pressure, will also be recorded. This is exactly the same information you normally supply to us when we see you in this clinic.

The next step is for blood tests, which you have already come for. In addition to the two usual blood tubes we take, we will add two extra tubes which will be taken to different laboratories to be used to do exactly the same test you have come for, testing CD4 ("soldiers") as we say in our language. For completeness, each of the blood samples will be split into two for the purposes of this research.

#### **4) RISK AND DISCOMFORT INVOLVED.**

The only possible risk and discomfort involved is the taking of blood from a vein and the measurement of your vitals. Taking blood from a vein usually cause some minor discomfort and rarely pain. In addition a small bruise or a hematoma, small blood clot, may form on the puncture site. Extremely rarely, there are reports of a few people who fainted (syncope) during the procedure. I will be readily next to you to avoid any serious complications.

#### **5) POSSIBLE BENEFITS OF THIS STUDY.**

Many of these tests are done routinely on patients. It will enable us to treat you if you should have problems. So there are no added risks apart from the minor risks which one would have encountered during collection of blood.

- 6) I understand that if I do not want to participate in this study, I will still Receive standard treatment for my illness.
- 7) I may at any time withdraw from this study.

8) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2000), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

9) INFORMATION

If I have any questions concerning this study, I should contact:

- Dr Mlawanda. tel : 3134462 or cell: 6113351
- Dr Radebe tel: 3134804 or cell: 6067970

10) CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a fashion that patients remain unidentifiable.

11) CONSENT TO PARTICIPATE IN THIS STUDY.

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information has been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management in any way.

I hereby volunteer to take part in this study.  
I have received a signed copy of this informed consent agreement.

.....  
Patient / Guardian signature Date

.....  
Person obtaining informed consent Date

.....  
Witness Date

**VERBAL PATIENT INFORMED CONSENT** (applicable when patients cannot read or write)

I, the undersigned, Dr ....., have read and have explained fully to the patient, named .....and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his/her illness. The patient indicated that he/she understands

that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her treatment.

I hereby certify that the patient has agreed to participate in this study.

Patient's Name

(Please print)

Investigator's Name

(Please print)

Investigator's Signature

Date

Witness's Name Witness's Signature

(Please print)

Date

(Witness - sign that he/she has witnessed the process of informed consent)

#### **Appendix 4: WHO Guidelines for initiating ART**

*Recommendation 1: WHO November 2009 Rapid Advice*

*When to start HAART in adults*

1. Start antiretroviral treatment in all patients with HIV who have CD4 count <350 cells/mm<sup>3</sup> irrespective of clinical symptoms. *(Strong recommendation, moderate quality of evidence)*
2. CD4 testing is required to identify if patients with HIV and WHO clinical stage 1 or 2 diseases need to start antiretroviral treatment. *(Strong recommendation, low quality of evidence)*
3. Start antiretroviral treatment in all patients with HIV and WHO clinical stage 3 or 4 irrespective of CD4 count. *(Strong recommendation, low quality of evidence)*

## Appendix 5: Correlation of Complications of HIV with CD4 counts

CD4 Counts	Infectious Complications	Non-Infectious Complications
>500/mm <sup>3</sup>	Acute retroviral Syndrome Candida Vaginitis	Persistent Generalised Lymphadenopathy Gullain-Barre Synd. Myopathy Aseptic Meningitis
200 – 500/mm <sup>3</sup>	Pneumococcal and Other Bacterial Pneumonia Pulmonary Tuberculosis Herpes Zoster Oral thrush Cryptosporidiosis Kaposi sarcoma Oral hairy leukoplakia	Cervical intraepithelial Neoplasia Cervical cancer B-cell lymphoma Anemia Mononuclear multiplex Idiopathic Thrombocytopenic Purpura Hodgkins lymphoma Lymphocystis Interstitial Pneumonitis
<200/mm <sup>3</sup>	Pneumocystis Carinii Pneumonia Disseminated Histoplasmosis and Coccidioidomycosis Milliary TB Extra-pulmonary TB Progressive multifocal leukoencephalopathy	Wasting Peripheral neuropathy HIV-associated Dementia Cardiomyopathy Vacuolar myelopathy Progressive Polyradiculopathy Non-Hodgkins lymphoma
<100/mm <sup>3</sup>	Disseminated herpes simplex Toxoplasmosis Cryptococcosis Cryptosporidiosis Microsporidiosis Candidal esophagitis	
<50/mm <sup>3</sup>	Disseminated Cytomegalo virus Disseminated Mycobacterium Avium complex	Central nervous System lymphoma

## Appendix 6: WHO Clinical Staging Of HIV-Related Disease In Adults

### Clinical Stage 1(Asymptomatic)

Persistent Generalised Lymphadenopathy

### Clinical stage 2 (Mild disease)

Unexplained moderate weight loss (<10% of presumed or measured body weight)  
 Recurrent respiratory tract infections (sinusitis, tonsillitis, otitis media and pharyngitis)  
 Herpes zoster  
 Angular cheilitis  
 Recurrent oral ulcerations  
 Papular Pruritic Eruption (PPE)  
 Seborrhoeic dermatitis  
 Fungal nail infections

### Clinical stage 3 (Moderate disease)

Unexplained severe weight loss (>10% of presumed or measured body weight )  
 Unexplained chronic diarrhoea for longer than one month  
 Unexplained persistent fever (above 37.5 °C, intermittent or constant, for > one month)  
 Persistent oral candidiasis  
 Oral hairy leukoplakia (OHL)  
 Pulmonary Tuberculosis(TB)  
 Severe bacterial infections (such as pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia)  
 Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis  
 Unexplained anaemia (<8 g/dl ), neutropenia (<0.5 x 10<sup>9</sup> /litre) or chronic thrombocytopenia (<50 X 10<sup>9</sup> /litre<sup>3</sup>)

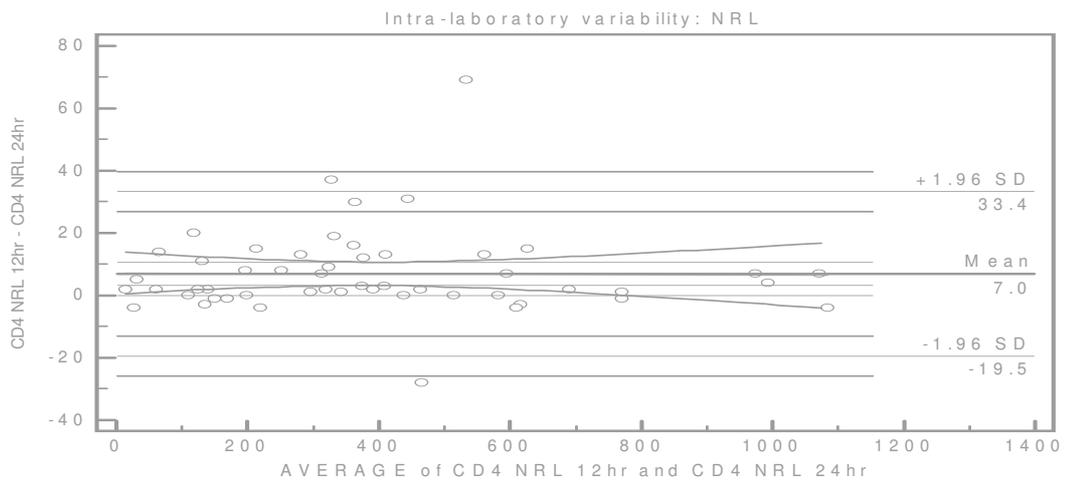
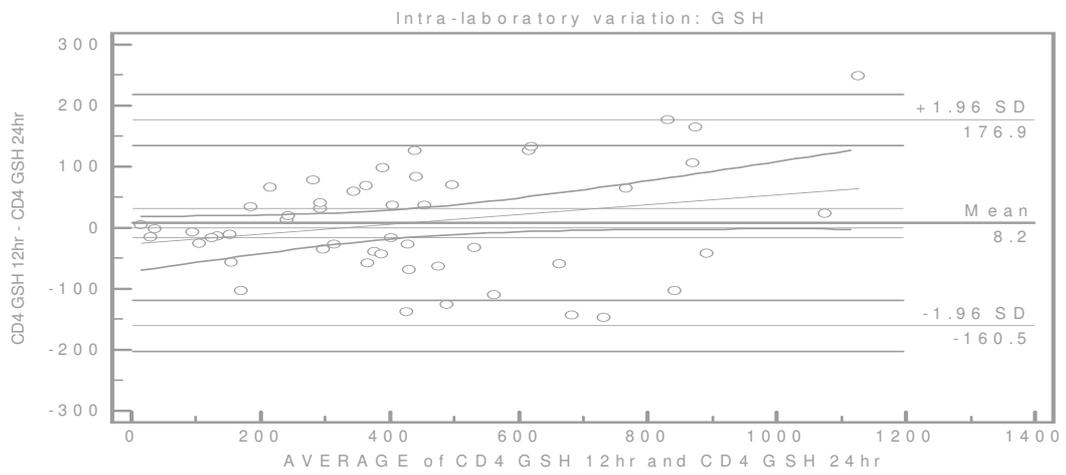
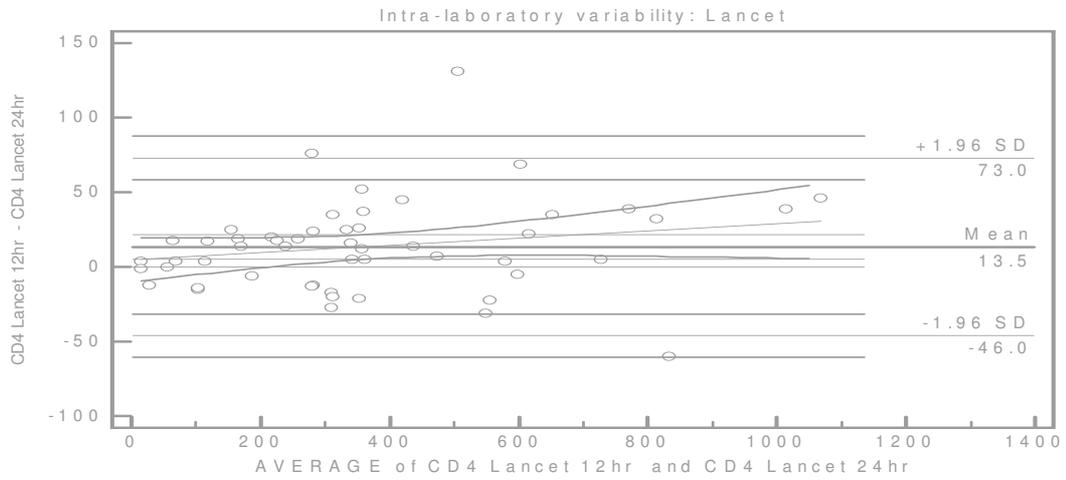
### Clinical stage 4 (Severe disease)

HIV wasting syndrome  
 Pneumocystis jiroveci pneumonia (PCP)  
 Recurrent severe bacterial pneumonia  
 Chronic herpes simplex infection (orolabial, genital or anorectal, of more than one month's duration or visceral at any site)  
 Oesophageal candidiasis (or candidiasis of the trachea, bronchi or lungs)  
 Extrapulmonary TB (EPTB)  
 Kaposi sarcoma  
 Cytomegalovirus (CMV) infection (retinitis or infection of other organs)  
 Toxoplasmosis of the central nervous system (CNS)  
 HIV encephalopathy  
 Extrapulmonary cryptococcosis including meningitis  
 Disseminated non-tuberculous mycobacterial infection  
 Progressive multifocal leukoencephalopathy (PML)  
 Penicilliosis; Chronic cryptosporidiosis; Chronic isosporiasis  
 Disseminated mycosis (extrapulmonary histoplasmosis, coccidioidomycosis)  
 Recurrent septicaemia (including due to non-typhoidal Salmonella)  
 Lymphoma (cerebral or B-cell, non-Hodgkin)  
 Invasive cervical carcinoma  
 Atypical disseminated leishmaniasis  
 Symptomatic HIV-associated nephropathy or HIV-associated  
 Cardiomyopathy

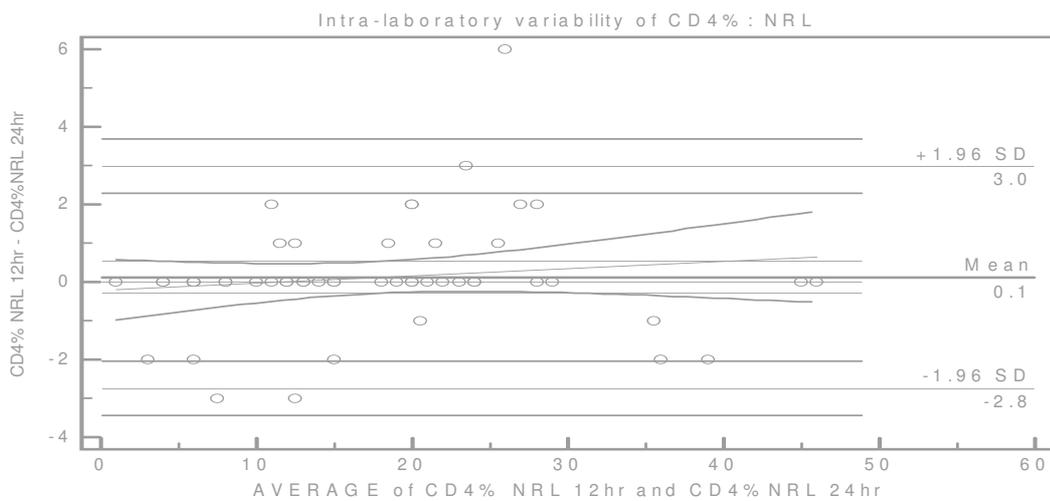
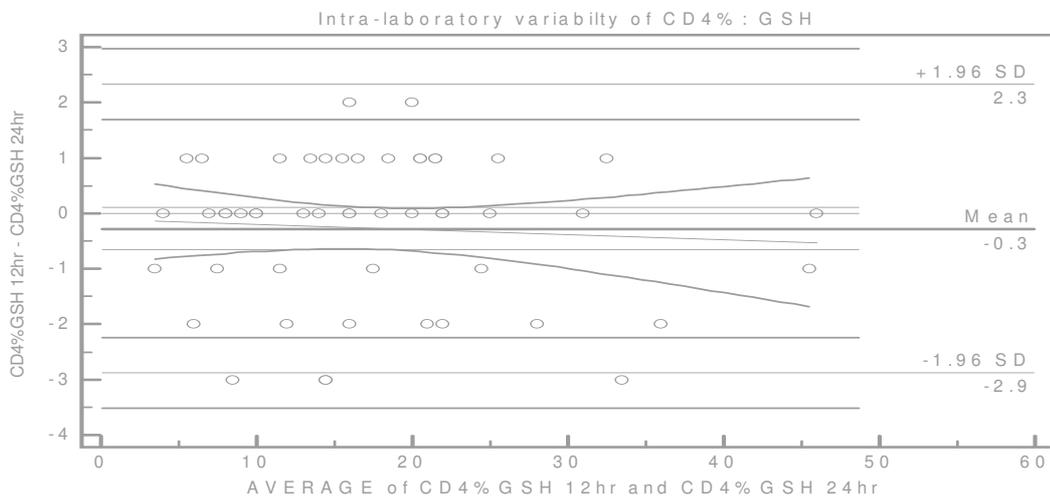
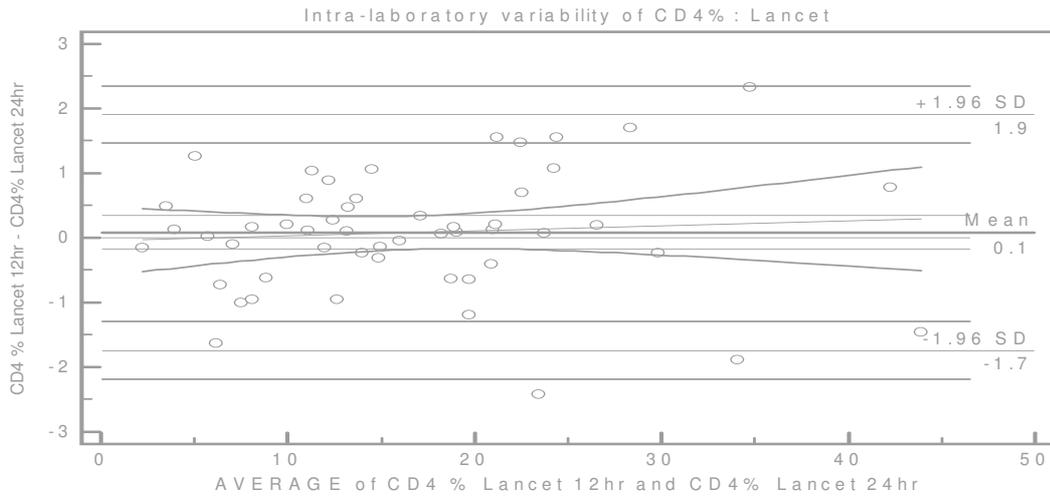
**Appendix 7: Byrt's criteria for assessing Kappa strength**

0.93 to 1 :	excellent agreement
0.81 to 0.92:	very good agreement
0.61 to 0.80:	good agreement
0.41 to 0.60:	fair agreement
0.21 to 0.40:	slight agreement
0.01 to 0.20:	poor agreement
<0.00 :	no agreement

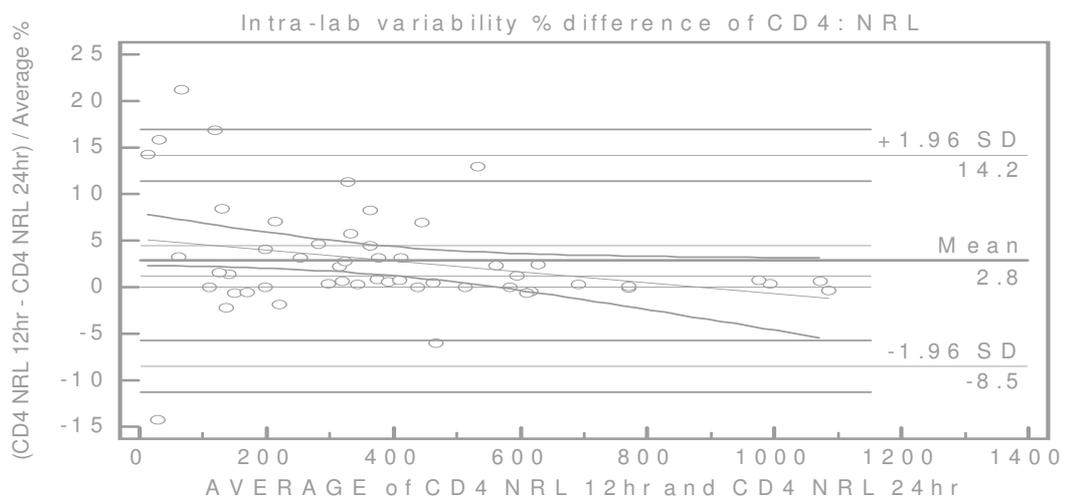
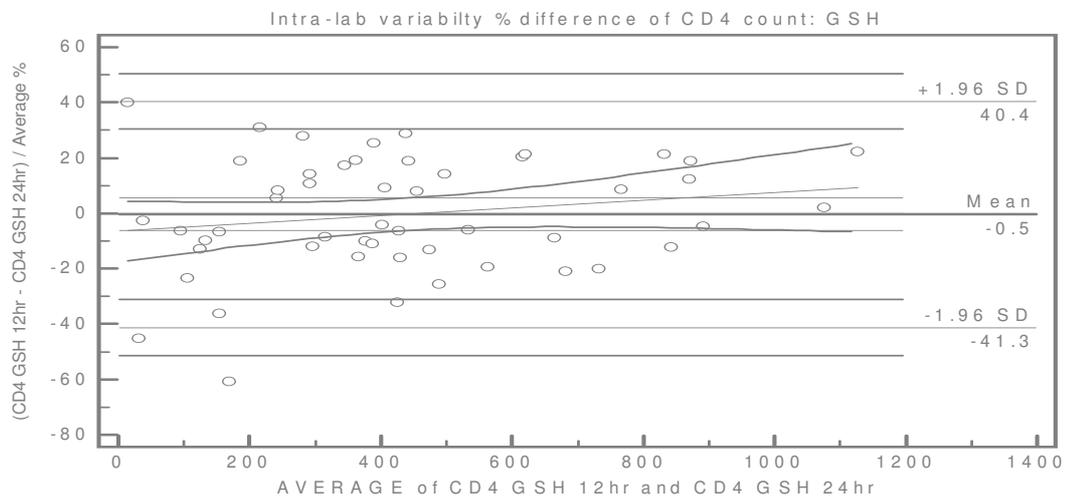
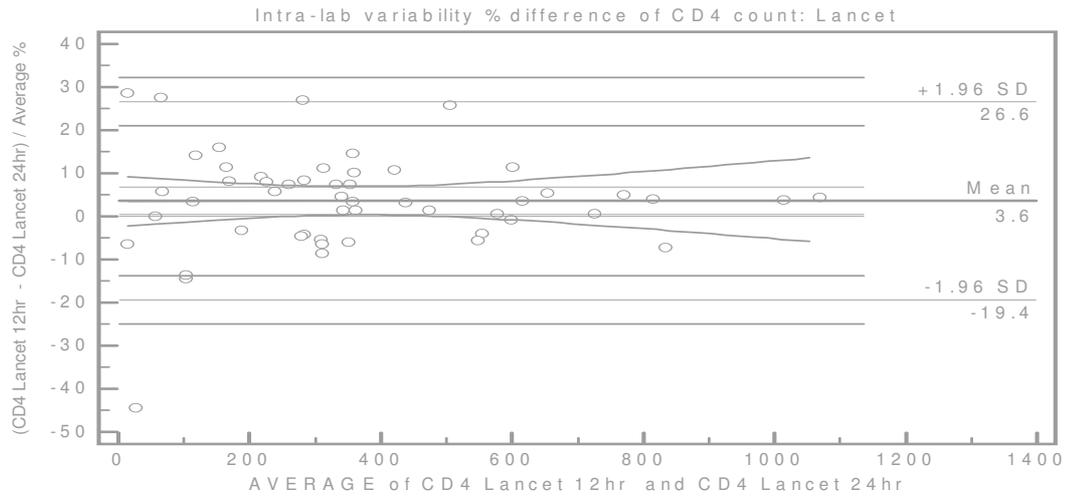
**Appendix 8: BA plot for intra-laboratory variability of CD4 count: Lancet, GSH and NRL**



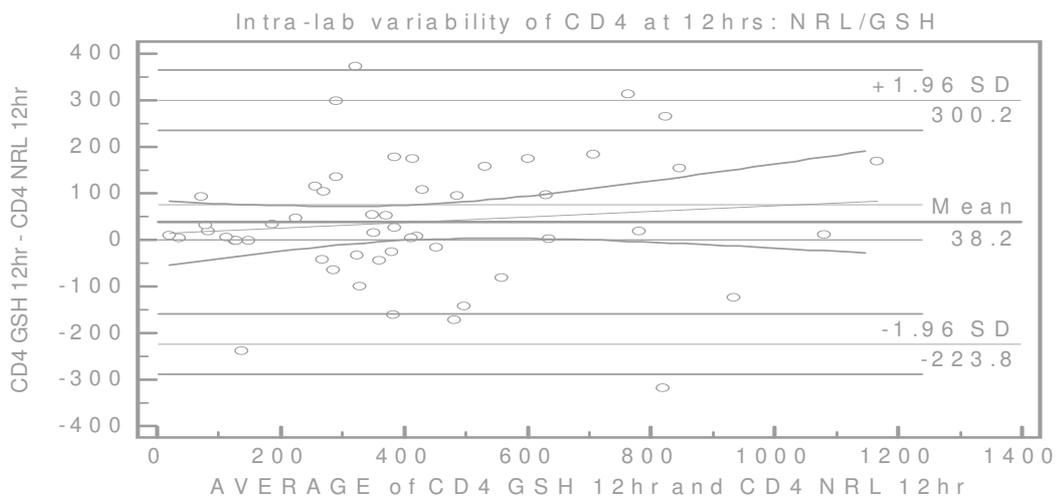
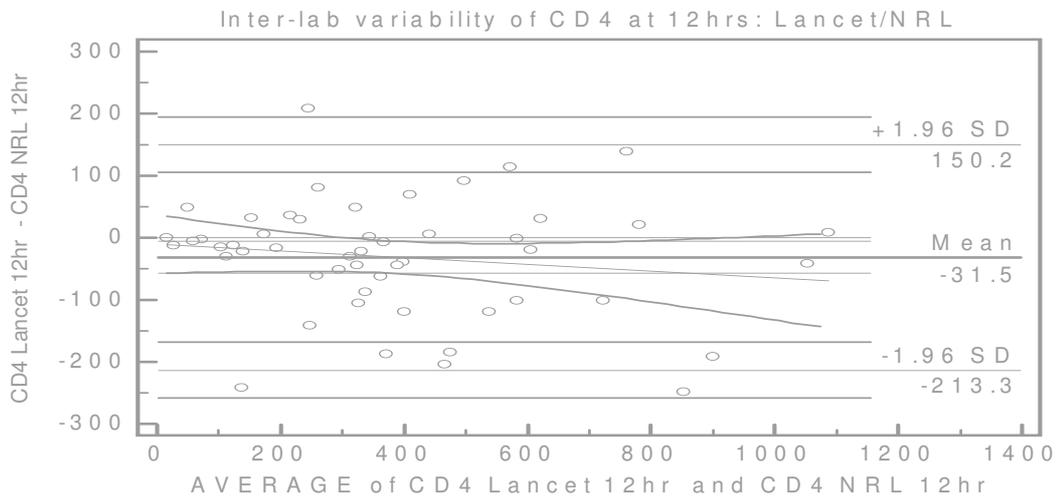
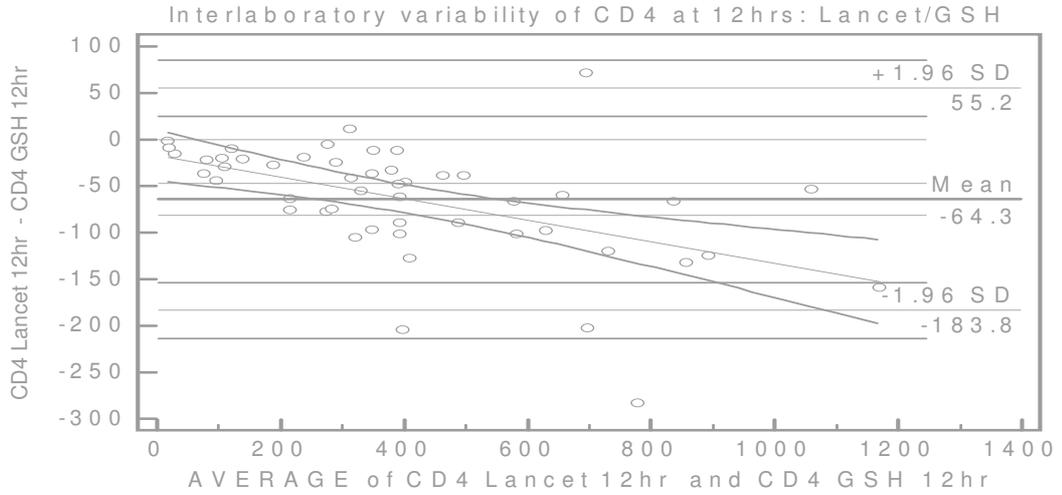
**Appendix 9: BA plots for intra-laboratory variability of CD4%: Lancet, GSH and NRL**



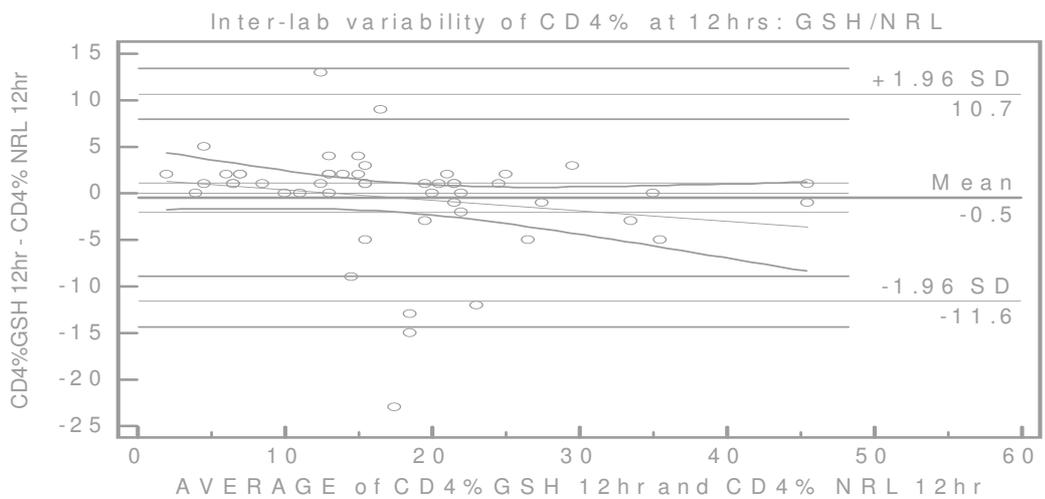
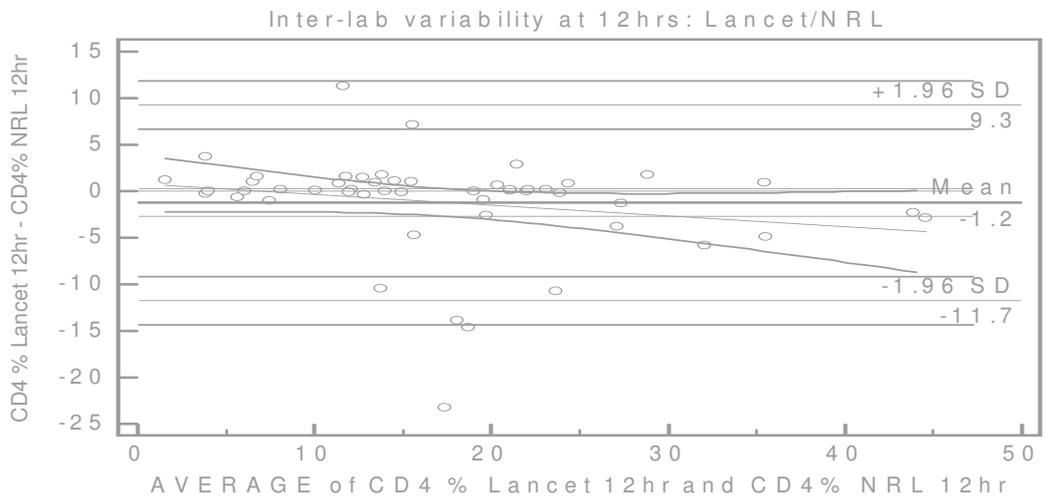
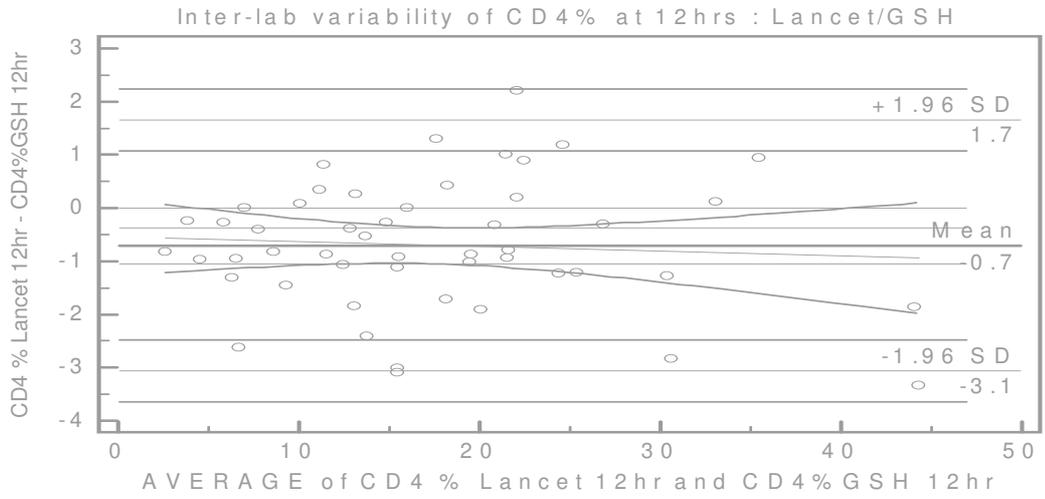
**Appendix 10: BA plots for intra-laboratory variability %difference of CD4: Lancet, GSH and NRL**



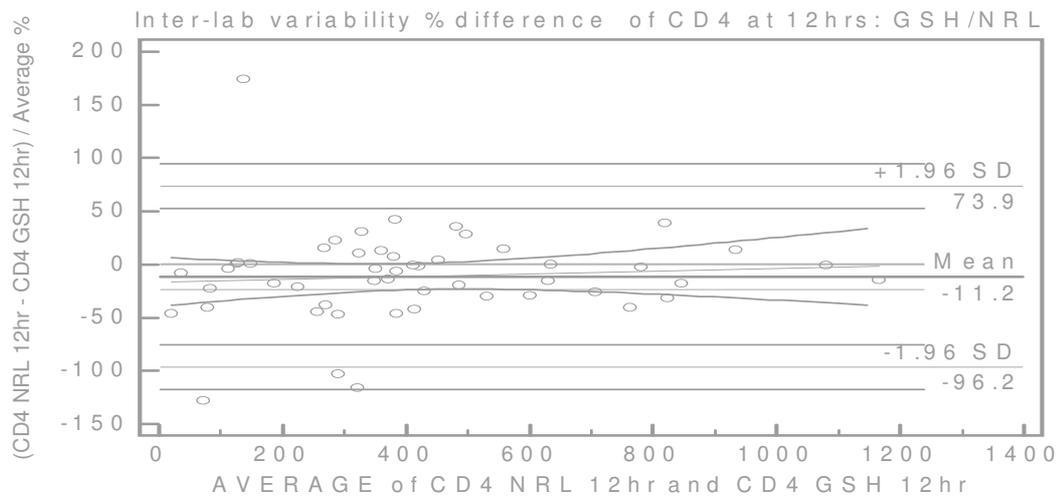
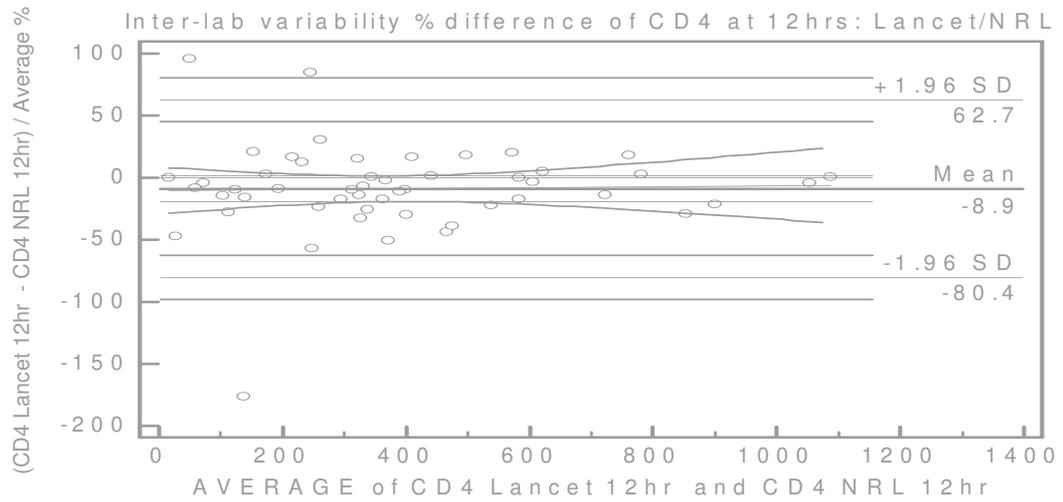
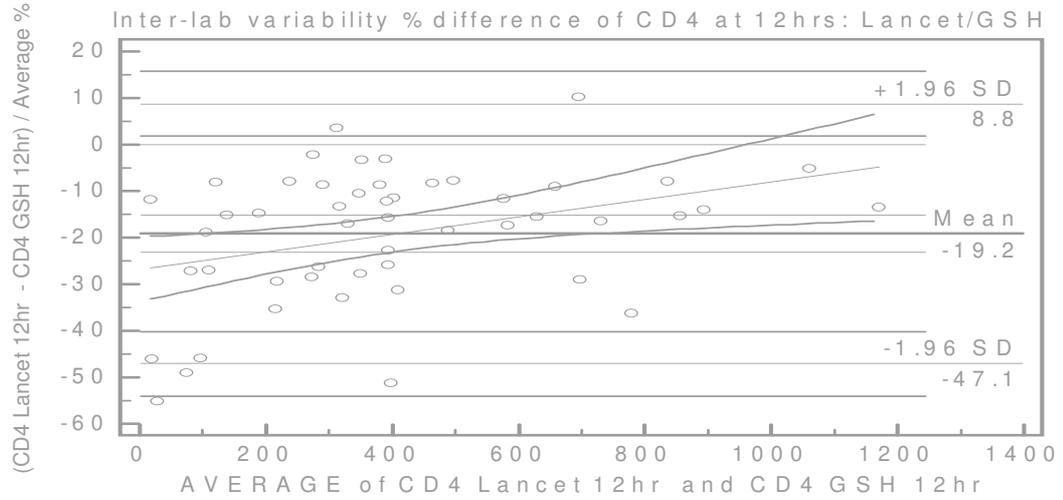
**Appendix 11: BA plots for inter-laboratory variability of CD4 at 12hours: Lancet/GSH, Lancet/NRL and NRL/GSH**



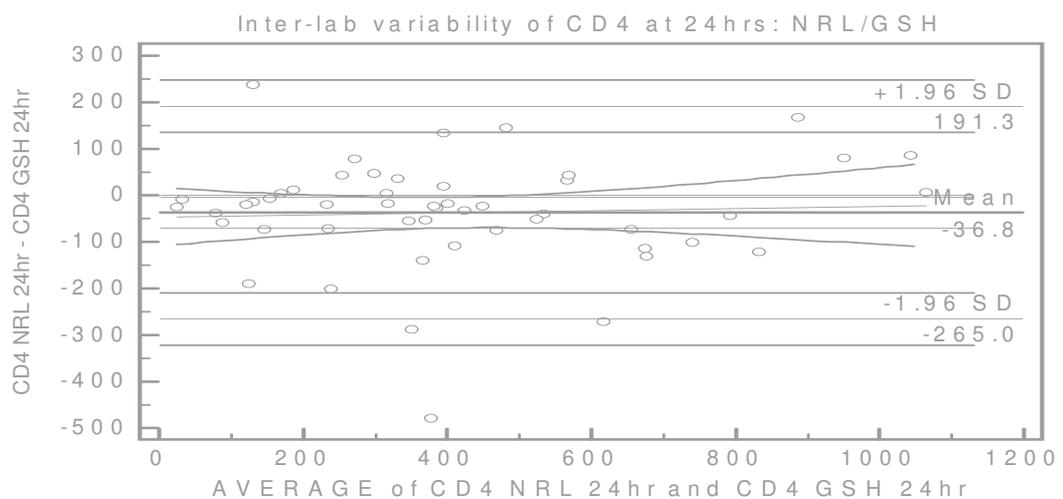
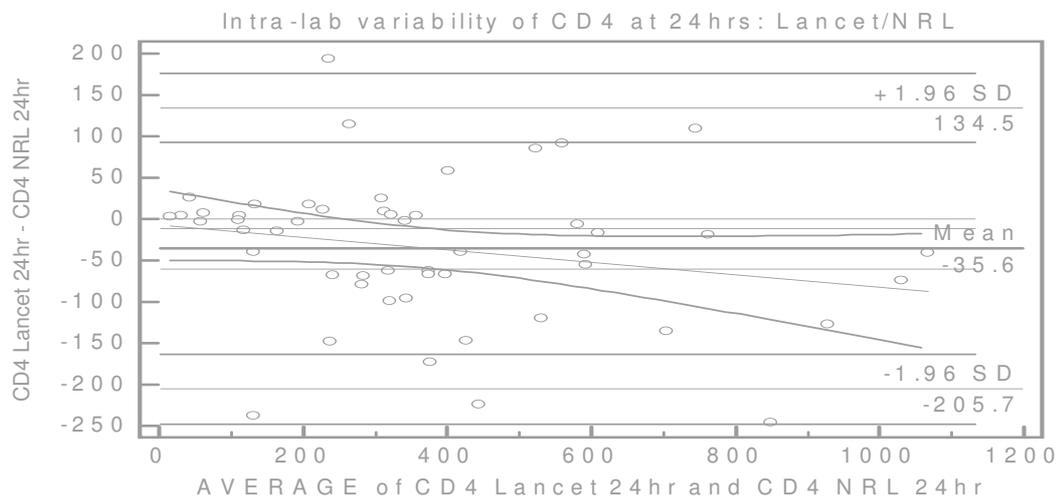
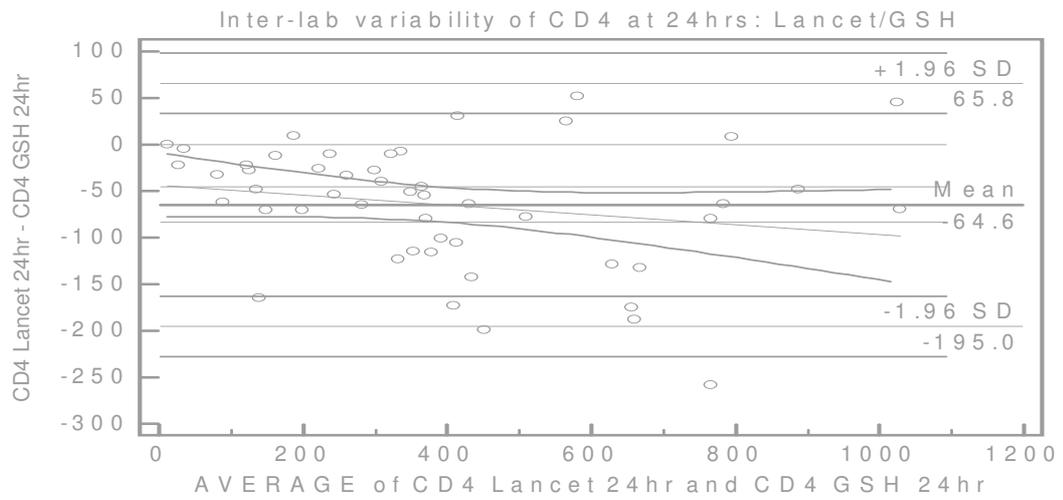
**Appendix 12: BA plots for inter-laboratory variability of CD4% at 12hours: Lancet/GSH, Lancet/NRL and NRL/GSH**



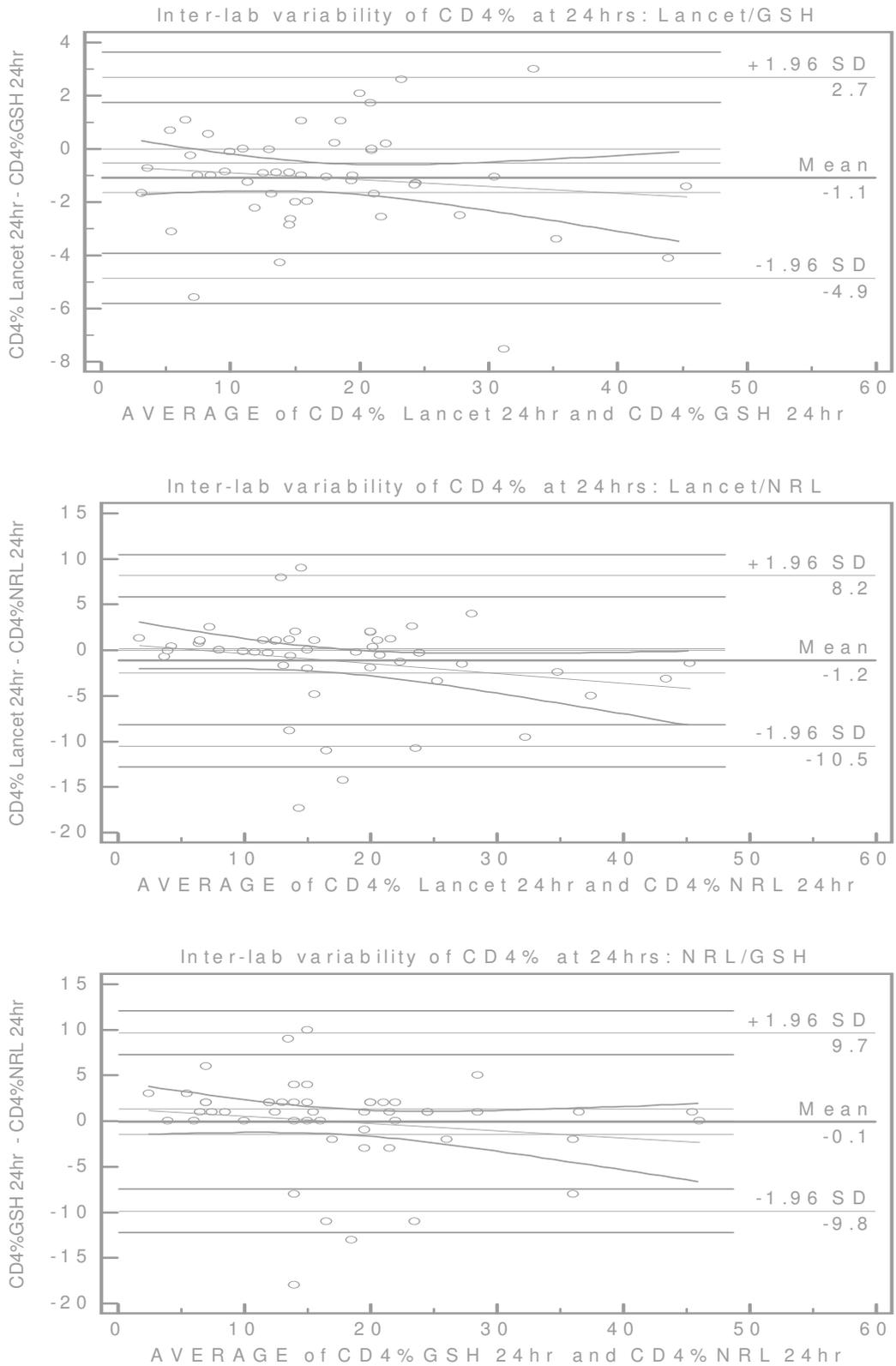
**Appendix 13: BA plots for inter-laboratory % difference of CD4 at 12hours: Lancet/GSH, Lancet/NRL and NRL/GSH**



**Appendix 14: BA plots for inter-laboratory variability of CD4 at 24hours: Lancet/GSH, Lancet/NRL and NRL/GSH**



**Appendix 15: BA plots for inter-laboratory variability of CD4% at 24hours: Lancet/GSH, Lancet/NRL and NRL/GSH**



**Appendix 16: BA plots for inter-laboratory % difference of CD4 at 24hours: Lancet/GSH, Lancet/NRL and NRL/GSH**

