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# Analysis of gamma-delta T cells in black South African patients with active tuberculosis

**Sedick, Qanita**

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**LIST OF CORRECTIONS EXAMINER 1:**

**INTRODUCTION:**

This is an interesting and relevant area of research, with potential basic science and diagnostic applications. Although there are international data published on this topic, local information is lacking particularly in the setting of high HIV and TB prevalence. Despite the potential, this research report has been compromised by unacceptable and major errors. As the report currently stands it is inadequate for partial fulfilment for the award of the MMed degree. The errors are of a nature which would require substantial amendments and re-examination by the examiner.

Thank you for the valuable comments and opportunity to correct and improve this manuscript. Each comment from the examiner has been noted and corrected in the manuscript as outlined below:

**MAJOR CORRECTIONS**

1. INCLUSION OF IDENTIFYING DATA OF PARTICIPANTS:

Appendix 3: The candidate has included filled in and signed informed consent forms from both a normal control and a patient who participated in the study. These clearly identify the names of both of the participants and the hospital number of the patient concerned.

This is absolutely unacceptable and raises serious doubt that the candidate understands the ethical aspects of this project.These documents need to be removed and the candidate reminded of the ethical obligations of research. Please also include the original ethics clearance certificate in Appendix 6, not only the approval for a protocol amendment.

Appendix 3 was a demonstration for the submission of this report and the brackets which indicates control and patients were added by me for the purpose of this submission to illustrate that controls and patients were interviewed. I understand the ethical concerns raised by the reviewer and acknowledge that the names of the participants should not have appeared in this manuscript. The actual collection of data was done with extreme discretion. The original signed copies of consent forms have been removed and replaced

with blank forms (Appendix 3) to illustrate the consent form. All individuals (controls and patients) signed informed consent.

The original ethics clearance certificate and relevant documentation have been included (Appendix 6).

2. DISCREPANCIES BETWEEN DATA PRESENTED IN A TABLE IN APPENDIX 8 AND THAT DISCUSSED IN THE BODY OF THE REPORT:

A table with data on all subjects participating in the study is included in appendix 8. This is the only demographic data presented and is not discussed in any depth in the research report. It is, however, extremely worrying that the data in this table does not appear to correlate with the information discussed in the text and/or is very ambiguous. This makes it extremely difficult for the examiner to assess the quality or value of the data presented or the statistical analysis.

The demographic sheet has been revised and clarified according to original data collection sheets (Appendix 8).Please see further comments addressed below.

As an example, on page 22, the candidate states that 17 control samples were included in the study. According to the text, 15 patients agreed to voluntary HIV testing and 2 refused consent. 1 control proved to be HIV positive. In Appendix 8 this information is recorded as 4 controls that did not consent to HIV testing and 2 controls do not have their HIV status documented. Further, on pages 22 and 42, it is stated that all 17 cases were included in the study / in the data analysis, but on page 29 the candidate writes that control cases

A14/A15/A1/A2/A3 and A4 were excluded. So exactly how many controls were included in the study? Was the control subject who proved to HIV positive excluded from statistical analysis? Similar discrepancies and ambiguities are noted for both the patient groups as well.

The materials and methods section 2.1 has been carefully reviewed and altered. Please see the changes made below which have been bolded.

‘The study was a prospective study which was performed over a period of 2 months**. A total number of 49 samples were collected**. Samples from patients were collected at the Charlotte Maxeke Johannesburg Academic Hospital from 1 July 2011 until 31 August 2011. Samples were analyzed at the National Health Laboratory Services, department of Molecular Medicine and Haematology based at the Charlotte Maxeke Johannesburg Academic Hospital.

Seventeen control samples were collected in ethylene diamine tetra-acetic acid (EDTA) anti-coagulated tubes from health care workers at the Charlotte Maxeke Johannesburg Academic Hospital. Participants signed informed consent for blood to be used for the study. Controls were given the option of free HIV testing with appropriate counseling by independent counselors not involved with the study. Those that consented were taken to an HIV testing centre where a rapid HIV test was performed**. Seventeen control patients were enrolled of which four (A6, A8, A12, A13) refused consent for the HIV testing but were included in the study. One control tested HIV positive. This patient was subsequently referred to the HIV clinic and was excluded from the study. During processing of samples, further exclusion of samples was required. This resulted in samples A1, A2, A3 and A4 being excluded. These samples were excluded because the antibody we used to mark gamma-delta T cells for this batch was found to stain sub-optimally. Samples A14, A15 were also excluded from our analysis because there were too few events noted on these sample plots. Therefore, 10 control samples were analyzed in total.**

Patient samples were collected in two groups. The first group included patients that were HIV positive and had no symptoms or signs of current Tuberculosis. **TB microscopy was not collected routinely on this patient group and exclusion of Tuberculosis was based on absence of** symptoms and signs of Tuberculosis. Symptoms for which patients were screened included productive cough, night sweats and loss of weight. Signs included pleural effusions and signs of immunosuppression such as cachexia. Data was collected on a patient data sheet (Appendix 4). These samples were obtained from the Charlotte Maxeke Johannesburg Academic hospital HIV outpatient clinic. **A total number of seventeen patients were collected in this group. During sample processing, it was found that B5 had too few cells for analysis and was thus excluded from the analysis. A total of 16 samples in this group were thus used in the final analysis.**

The second group included patients that were HIV positive with recent onset (previous two weeks) of smear positive Tuberculosis and that had only been on **anti Tuberculosis treatment for less than 4 days or had not started anti Tuberculosis treatment.** These samples were obtained from the medical and infectious disease wards at the Charlotte Maxeke Johannesburg Academic Hospital. The sputum results for smear positivity of each patient were obtained from the microbiology laboratory. We excluded all patients who had been on anti-Tuberculosis therapy for longer than 4 days and who did not have smear-positive sputum result. There were no pregnant women in our cohort. The age range for all patients and health care workers was a minimum of 18 years with no upper age limit. **A total number of fifteen patients in this group were collected. There were**

**no exclusions in this group. Therefore, all the samples in this group were used in the analysis.'**

In addition, there are apparent discrepancies in methodology between appendices and that discussed in the research report. The table in appendix 8 suggests that the normal control and HIV+/TB- control groups all had negative TB microscopy. This is not included in the materials and methods section. Was TB microscopy performed on controls and if so on what sample? On page 22 – the candidate states that TB patients who had been on TB therapy for longer than a week were excluded; patient data sheet in Appendix 4 excludes patients on therapy for 4 days.

Please note that Group A and B patients did not have TB microscopy performed. This should not have been recorded as negative in the patient demographic sheet. It should have been preferably left blank or not applicable. This was a notation error and has been corrected in the data sheets as "not applicable". No Tuberculosis patients had been on TB therapy for longer than 4 days.

All this data needs to be reviewed and clarified. It should be absolutely unambiguous as to how many patients were initially included in each of the 3 study groups, how many subjects were excluded and why and results from how many subjects were used in the statistical analysis. This could be done in a table format for each subgroup in the body of the report or clearly documented in the text. The methodology should also be absolutely unambiguous and accurately reflect the methodology of the project.

**Enrollment and exclusion numbers are summarized in the table below which has been added to Section 2.1**

| | GROUP A (CONTROLS) | GROUP B (HIV+TB-) | GROUP C (HIV+TB+) |
|---|---|---|---|
| TOTAL NUMBER SAMPLES COLLECTED | 17 samples | 17 | 15 |
| EXCLUSIONS | A1, A2, A3, A4 excluded due to poor staining<br><br>A7 excluded due to HIV positivity<br><br>A14, A15 excluded due to too few cells for analysis | B5 excluded due to too few cells for analysis | No exclusions in this group |
| FINAL SAMPLES ANALYSED | 10 samples | 16 samples | 15 samples |

3. FAILURE TO ANALYSE AND / OR DISCUSS ANY OF THE DEMOGRAPHIC DATA PRESENTED IN APPENDIX 8:

Refer to PAGE 30: SECTION 3. RESULTS: This section needs to start with an overview of the study population and the demographics of the patients included. The raw demographic data is presented in Appendix 8, but never referred to in the text. There appears to be no statistical analysis of any of the demographic data at all – descriptive statistics, at the very least, should be included. These may be more easily presented in one or more tables.

The following was added under Section 3 to address these issues.

'The study population included black South African patients from the HIV clinic and respiratory wards at the Charlotte Maxeke Johannesburg Academic Hospital. Appendix 8 shows the patient demographic profiles. A comparison with respect to age, gender, HAART therapy and CD4 counts was done using a uni-variate analysis on Graph-Pad Prism software (La Jolla, California).' See continuation of descriptive statistics under the next point.

Each of the 3 study groups should be assessed for Male: Female ratios of participants and with descriptive statistics of age of patients in each group. For the HIV+ patients, in addition, there should be reporting of descriptive statistics of CD4 count and viral load and an assessment of the % patients in each group on HAART. Statistical analysis between the groups is also suggested – could differences in sex ratio, HAART therapy or age have impacted on your results? The potential needs to be acknowledged and discussed in your discussion. On page 40 – the candidate has referenced an article showing that HAART may partially correct the skewing of the V gamma 2 T-Cell receptor repertoires (Bordon J et al, 2004) – this needs to be discussed with reference to the results presented in the research report.

Gender differences were evaluated in the cohort. There was a total number of 18 males and 31 females in the cohort. There were 4 males and 13 females in group A. There were 8 males and 9 females in group B. There were 6 males and 9 females in group C. Differences in gender was analyzed using a Chi Square test. No difference with respect to gender was found between the groups ($p=0.36$) (table 6).

**Table 6: Gender differences in the cohort**

| | GROUP A | GROUP B | GROUP C | TOTAL NUMBER |
|---|---|---|---|---|
| **MALES** | 4 (23% of group A) | 4 (30% of group B) | 6 (40% of group C) | 18 (36% of total) |
| **FEMALES** | 13 | 9 | 9 | 31 |
| **TOTAL NUMBER** | 17 | 13 | 15 | 49 |

All three groups were assessed for age differences. The median age in group A was 40 years, in group B was 38 years and in group C was 34 years. The youngest person was 20 years old in group A and group C. The oldest person was 84 years old in group A. Age differences were analyzed using a Chi-square test. There was no difference found between the groups with respect to age (p=0.292) (table 7).

**Table 7: Age differences in the cohort**

| | GROUP A | GROUP B | GROUP C |
|---|---|---|---|
| **MINIMUM AGE (YEARS)** | 20 | 31 | 20 |
| **MEDIAN AGE (YEARS)** | 40 | 38 | 34 |
| **MAXIMUM AGE (YEARS)** | 84 | 71 | 52 |

CD4 counts were not measured in the healthy controls. In the HIV infected groups, the minimum CD4 count was 10 x 10^6/l in group C and the maximum CD4 count was 739 x 10^6/l in group B. Using a Mann Whitney test, the median CD4 count (median=90 µl) was statistically lower than the median CD4 count in group B (median=465 µl) (p=0.001) (table 8).

**Table 8: CD4 counts in the cohort**

| UNITS (X 10^6/L) | GROUP B | GROUP C |
|---|---|---|
| **MINIMUM** | 45 | 10 |
| **MEDIAN** | 465 | 90 |
| **MAXIMUM** | 739 | 477 |

In group B, 15 patients were on HAART and 2 patients were not on HAART. In group C, 7 patients were on HAART and 8 patients were not on HAART. Using a 2 tailed fishers exact test, there was a significant difference in the proportion of patients receiving HAART therapy and those not on HAART therapy (p=0.021). Table 9 shows the number of patients on HAART therapy for each group.

**Table 9: Number of patients on HAART therapy**

| | HAART THERAPY |
|---|---|
| **GROUP B** | 15 patients on HAART, 2 patients not on HAART |
| **GROUP C** | 7 patients on HAART, 8 patients not on HAART |

4. INADEQUATE REFERENCING:

The referencing of this research report is absolutely unacceptable and the referencing needs to be revised completely, using importation from a referencing package (such as footnote) and a standardized referencing format. It is virtually impossible for a reader to access some of the references, based on inaccuracies in the information supplied.

The referring has been completely revised using the software Endnote and in the Vancouver style.

Some examples:

a. Referencing style is not standardized in the research report or the reference list, with some references even lacking important information such as the year of publication, volume or pages.
b. The reference list is alphabetized; however, some of the references are not in the correct alphabetical order making it difficult to locate references.
c. There are numerous errors within references. Some examples:
    - Ahmad et al, 2011 – cited on pages 1 and 2 is not in the list of references. Does this refer to Ahmad S, 2011 in the reference list? This is a repeated problem with authors referenced as et al in the text, but with a single author listed in the reference list – other examples Cardona, 2007/2009; Jo,2008; Kusner,2005 etc).
    - Incorrect information in the references - Moru, 2010 is referenced in the abstract, however, when accessing the complete article this paper appears to have been written by Lange C & Mori T.
    - Some references in the text are absent from the reference list (eg Harding et al, 2010; Bowdish et al,2009; Chan et al, 1992; Gold, 2012)
    - Book chapters and electronic references need to be reviewed.
    - Referencing needs to be updated to include the most recent information available.

The following changes were made in response to these comments:

**a.** The referencing style was altered to the Vancouver method of referencing using the software Endnote.
**b.** All references have been alphabetized
**c.** The reference previously cited as Ahmad et al, 2011 was referring to Ahmad S, 2011. The same applies to the other authors mentioned such as Cardona PJ, 2007/2009, Jo, 2008 and Kusner DJ, 2005. References have been revised using Endnote.
**d.** The reference previously cited as Moru is now correctly references as Lange C & Mori T using Endnote (see reference number 68).
**e.** The references Harding et al, 2010, Bowdish et al, 2009, Chan et al, 1992 and Gold, 2012 have been added to the reference list using endnote.
**f.** New updated references have been added. These include the following as numbered in the reference list: Reference number 1, 2, 38, 39, 76, 80, 82, 90, 91, 101, 102, 103 and 105.
**g.** The first two sentences of the discussion have also been referenced-see reference number 70.
**h.** The most recent information regarding gamma delta T cells has been added to the text and highlighted in yellow throughout the corrected report.

5. CORRECTION OF FORMATTING AND STRUCTURAL ERRORS:

Page numbers in the index and the lists of tables and figures are incorrect (see pages V, VI, X-XI). In the list of figures, some of the figures are recorded twice (Figures 11 and 12 are listed with the same heading); some of the figures are not in the research report (there is no Figure 11 in the report); and some of the headings / content are discrepant between the list of figures and the actual figures (Figure 12).

At the stage of submission to an examiner, it is expected that these types of errors will have been corrected and the candidate needs to review and correct these aspects of this research report.

The index has been updated. Figures 1 up to and including figure 7 have been removed. There are now 8 figures in the report. Figure 11 is now Figure 4 and figure 12 is now figure 5. On page v, a list of figures now appears as follows:


| FIGURE NUMBER | TITLE OF FIGURE | PAGE NUMBER |
|---|---|---|
| Figure 1 | Spill over in AmCyan channel | 21 |
| Figure 2 | Spill over in PerCP CY5.5 channel | 22 |
| Figure 3 | Spill over of fluorochromes into CD8 Beta APC | 22 |
| Figure 4 | Box plots of gamma delta T cells in all patient groups | 29 |
| Figure 5 | Flow cytometric plots of gamma delta T cells | 30 |
| Figure 6 | Box plots of cytotoxic T cells | 31 |
| Figure 7 | Flow cytometric plots of helper and cytotoxic T cells | 32 |
| Figure 8 | Correlation graphs | 33 |

Table 3 up to and including table 7 have been added to the report. The list of tables appears on page VI is now as follows:


| TABLE NUMBER | TITLE OF TABLE | PAGE NUMBER |
|---|---|---|
| Table 1 | Panel of CD markers | 13 |
| Table 2 | Lineage description | 14 |
| Table 3 | Enrolment and exclusion numbers | 17 |
| Table 4 | Titration of CD8 Beta | 19 |
| Table 5 | Initial and new antibody panel | 20 |
| Table 6 | Gender differences in cohort | 25 |
| Table 7 | Age differences in cohort | 25 |
| Table 8 | CD4 counts in the cohort | 26 |
| Table 9 | Number of patients on HAART therapy | 27 |


6. COPYRIGHT FOR FIGURES:

Many of the Figures have been taken directly from internet websites (such as Wikipedia, CDC Public Health Image Library) or textbooks, such as Figures 1, 2, 3, 4, 5, 6 and 7. There could be copyright infringement issues with doing this. Many of these figures are non-essential to the report. I would suggest they are removed and replaced with Figures and Tables directly pertinent to the experimental work.

In reference to other figures and tables these need to be referred to and discussed in the text.

a. All internet and Wikipedia references have been removed along with figures 1 up to and including figure 7.
b. The list of figures has been updated as listed above. Tables 3 up to and including 7 have been added and appear as listed above. Reference to figures and tables has been made in the text.

**MINOR CORRECTIONS**

1. ABSTRACT page II – remove reference (Moru, 2010) in line 4 from the abstract.

This reference has been removed.

2. PAGE 2: Correct sentence, does not make sense–"Tuberculosis is a communicable disease which is spread by infected individuals with active pulmonary TB patients who reside in close proximity to other family and community members in conditions of overcrowding (Ahmad et al, 2011)".

This has been replaced with the following sentence on page 2:

'Tuberculosis is usually spread by individuals with active pulmonary TB. These Tuberculosis patients usually reside in close proximity to other family and community members, thus facilitating the spread of the disease.'

3. PAGE 3: Correct sentence, does not make sense – "Granulomas include this immune response".

This sentence has been replaced with the following:

'Granulomas represent a part of this immune response'

4. PAGE 10: FIGURE 5 – Correct Reassessment to Recombination: "**Reassessment** is random and differs from that of another T cell".

Figure 5 was removed as suggested under major corrections

5. PAGE 10: Correct molecularly to molecule – "Gamma-delta T cells do not require uptake,processing or intracellular loading on the MHC **molecularly**for antigen presentation and are dependent on cell to cell contact"

The word "molecularly" has been replaced by "molecule".

6. PAGE 22: Remove respiratory symptoms suggestive of Tuberculosis including. - "Signs included **respiratory symptoms suggestive of Tuberculosis including** auscultatory changes, pleural effusions and signs of immunosuppression such as cachexia.

This legend and figure has been removed from the text.

7. PAGE 22: MATERIALS AND METHODS – This section needs an introduction on study design and study period.

The paragraph below was added to section 2.1 (see page 15):

'The study was a prospective study which was performed over a period of 2 months. Samples from patients were collected at the Charlotte Maxeke Johannesburg Academic Hospital from 1 July 2011 until 31 August 2011. Samples were analyzed at the National Health Laboratory Services, department of Molecular Medicine and Haematology based at the Charlotte Maxeke Johannesburg Academic Hospital.'

8. PAGE 22: Data was collected on a patient datasheet – refer the reader to Appendix 4.

This was added in brackets in the text.

9. PAGE 23: 2.2 CD4 testing: clarify if CD4 testing was part of routine investigations of the patients or specifically performed for the study.

The following sentence was added:

'The CD4 testing was part of routine investigations performed on the patient.'

10. PAGE 23: 2.3 Peripheral blood mononuclear cell isolation – this paragraph needs to be restructured and clarified – it is not in chronological order.

The paragraph now reads as follows - see Section 2.3 on page 17:

'Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Ficoll Hypaque gradients. The PBMCs were spun at a speed of 3000 rcf (relative centrifugal force) and washed three times in phosphate buffered saline (PBS). An antibody cocktail was prepared which consisted of each appropriate volume of the nine antibodies multiplied by the number of samples for testing. The staining volume of each antibody was obtained by titration. Each sample was then stained using the antibody cocktail for each day, to minimize pipetting inaccuracies.'

11. PAGES 23,25: All references to manufacturers of instruments and reagents need to include the location – so LSRII flow cytometer (BD Biosciences, San Jose, CA), Table 3: page 25 – include manufacturers and locations for each fluorochrome used.

Please refer to Table 3 for inclusion of manufacturer locations for each fluorochrome. The LSR Flow Cytometer now includes the location (see table 5, page 20).

12. PAGE 24: Optimization of panel (titrations) – Refer the reader to Appendix 9. Include a proper title and explanation of the figures in Appendix 9 to assist the reader in understanding how the titration was performed.

The title 'Optimization of panel (titrations)' was changed to 'Titrations of antibodies'

The following paragraph was added to Section 2.4.3, page 19:

'When preparing a sample for flow cytometric analysis, titration of antibodies is essential to find the optimal concentration of antibody that approaches the saturation level of binding sites. This will ensure that a fluorescent signal emission is proportional to the antigen in the sample. CD4 Alexa700 and CD8 PerCP CY5.5 had been titrated for the initial antibody panel. As illustrated in Appendix 9, the titration of CD8 Beta was performed. A series of dilutions of the antibody was prepared as follows:'

**Table 4: Titration of CD8 Beta**

| TUBE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| VOLUME OF CD8 BETA(µL) | 0.625 | 1.25 | 2.5 | 5 | 7.5 | 10 | 20 | UNSTAINED TUBE |
| TOTAL STAINING VOLUME(µL) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |

13. PAGE 26: Refer the reader to Figures 8, 9 and 10 when describing the FMO experiments.

Figures 8, 9 and 10 are now figures 1, 2 and 3.This has been referred to accordingly in the text on pages 21 and 22.

14. PAGE 29: 2.6 Exclusions – these need to be incorporated into section 2.1 Study Samples (page 22) to assist the reader in clarifying exclusion of samples.

The sample exclusions noted in section 2.6 was added to sections 2.1 in an additional paragraph as follows:

‘During processing of samples, further exclusion of samples was necessary. This resulted in samples A1, A2, A3 and A4 being excluded. These samples were excluded because the antibody we used to mark gamma-delta T cells for this batch was found to stain sub optimally. We also excluded samples A14, A15 and B5 from our data because there were too few events noted on these sample plots. Therefore, 42 samples in total were analyzed.’

15. PAGE 33: FIGURE 13 – this figure should be referred to and explained in the text – under gating strategy on page 29.

Figure 13 is now figure 5. The following paragraph was added under the section 2.7 - gating strategy:

‘As depicted in figure 5, gamma delta TCR is plotted on the y-axis and VD2 TCR is plotted on the x–axis. The frequency of total gamma-delta T cells was calculated by adding the top two quadrants of each plot. The VD2 TCR subset was evaluated by adding the top and bottom right hand quadrants of each plot.’