Factors influencing CD4+ T cell counts in people living with HIV with end-stage kidney disease

1. **Melanie Pretorius**
   Student number: 1481305
   MBChB (Pretoria)
   Department of Haematology and Molecular Medicine
   National Health Laboratory Services Charlotte Maxeke Johannesburg Hospital
   University of Witwatersrand
   7 York Road
   Parktown, Johannesburg
   South Africa
2. **Estee Benade**
   MBBCh (Wits), Mmed (Wits), FCPath (Haem)
   FRCPC Hematopathologist Saskatchewan Health Autority
   +1 607 807 5750
   estee.benade@saskhealthauthority.ca
3. **June Fabian**
   MBBCh (Wits), FCP(SA), MMed (Wits), Cert Neph (SA)
   Donal Gordon Medical Centre
   june.fabian@mweb.co.za
4. **Denise Lawrie**
   MSc Haematology (Wits), PhD (Wits)
   Department of Haematology and Molecular Medicine
   National Health Laboratory Services Charlotte Maxeke Johannesburg Hospital
   University of Witwatersrand
   7 York Road
   Parktown, Johannesburg
   South Africa
   denise.lawrie@nhls.ac.za
5. **Elizabeth Mayne**
   BA (SA), MBBCh (wits), MMed (wits), FCPath (haem)
   Associate Professor and Head of Department: Immunology
   National Health Laboratory Services Charlotte Maxeke Johannesburg Hospital
   University of Witwatersrand
   (011) 489 8862
   7 York Road
   Parktown, Johannesburg
   South Africa
   elizabeth.mayne@nhls.ac.za

Candidate's Declaration

I, Melanie Pretorius, declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Masters of Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)

10 day of October 20 19 in Pretoria

Declaration: Student's contribution to article and agreement of co-authors

Declaration: Student's contribution to article and agreement of co-author(s)

I, **Melanie Pretorius (1481305)**, declare that this Research Report is my own work and that I contributed adequately towards research findings published in the article stated below which are included in my Research Report.

Signature of Student: MPretor. Date 18/9/2019

Name of Primary Supervisor Prof. Elizabeth Mayne

Signature of Primary Supervisor Date 18/09/2019

Agreement by co-authors:

By signing this declaration, the co-authors listed below agree to the use of the article by the student as part of her Research Report.

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Authors	Name	Signature	Date
1st Author	Dr. Melanie Pretorius	MPretor.	10/10/2019
2nd Author	Dr. Estee Benade		18 September 2019
3rd Author	Dr. June Fabian		01/10/2019
4th Author	Dr. Denise Lawrie	Denise Lawrie	26/9/2019
5th Author	Prof Elizabeth Mayne		18/09/2019

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Abstract

Introduction: In South Africa, it is estimated that ~7 million people are living with Human Immunodeficiency Virus (HIV). HIV is associated with an increased risk of kidney disease. For people living with HIV (PLWH) who develop end-stage kidney disease (ESKD), access to renal replacement therapy can be difficult. Kidney transplantation is a cost-effective option, with improved overall survival and better quality of life. Eligibility criteria for kidney transplantation in Johannesburg includes a sustained CD4+ T cell count of >200 cells/µl and suppressed HIV replication. This study aimed to investigate the influence of hemodialysis on the lymphocyte subsets in PLWH with ESKD.

Methods: Study participants and controls were recruited from renal dialysis centres in Johannesburg. Demographic data, social data, serial CD4+ T cell counts, serial HIV viral load measurements and blood samples were collected (before and after a haemodialysis session). Lymphocyte subsets were then measured.

Results: Our cohort showed a statistically significant increase in the post-dialysis % of CD4+ T cells and the absolute CD4+ T cell counts. The longitudinal trend analysis for the % of CD4+ T cells revealed a significant increase in five participants and a single patient had a significant decrease in the longitudinal trend analysis for the absolute CD4+ T cell counts. The longitudinal trend analysis for HIV viral load revealed the majority of our participants were not virologically suppressed.

Conclusion: This study showed that haemodialysis does not negatively impact CD4+ T cell count, suggesting that immunologic recovery is not impeded by treatment of the underlying ESKD.

Introduction

In South Africa, Human Immunodeficiency Virus (HIV) infection remains a leading healthcare concern. It is estimated that approximately 7 million people are infected. Of these 7 million HIV-infected patients, only 3,4 million patients are currently receiving combined antiretroviral therapy (ART). [1]

HIV infection leads to widespread immunological and subsequent organ dysfunction. End-stage kidney disease (ESKD) in HIV infection has been attributed to a number of causes including HIV-mediated renal damage, exposure to nephrotoxic agents including tenofovir disoproxil fumarate and presence of opportunistic infections. In patients on antiretroviral therapy (ART), with a reduction in opportunistic infections, there is a concomitant increased prevalence of non-communicable diseases including diabetes mellitus and hypertension in this population. (Table 1). [1-3]

Table 1: Causes of renal dysfunction in people living with HIV (PLWH)

Acute Kidney Injury (1-3)	Dehydration secondary to gastro-enteritis Sepsis and opportunistic infections (e.g. *Mycobacterium Tuberculosis*) HIV-associated thrombotic microangiopathies (e.g. TTP/HUS[1])
Chronic Kidney Disease (1-3)	Glomerular lesions • HIV-associated nephropathy (HIVAN) • HIV-associated nephropathy with focal glomerulosclerosis (HIV-FSGS) • HIV-immune complex deposition (HIVICD) • Other glomerulonephropathies (including amyloidosis, minimal change disease, immunotactoid nephropathy) Tubulointerstitial disease • Proximal tubular injury – Tenofovir toxicity • Chronic tubular injury – Amphotericin, tenofovir toxicity • Crystal nephropathy – Ciprofloxacin, Acyclovir (intravenous) • Interstitial nephritis – Infections (hepatitis B), immune reconstitution inflammatory syndrome following ART[2].
Co-morbid diseases (1-3)	Hypertensive nephrosclerosis Diabetic nephropathy Auto-immune disease (lupus nephritis)
Genetic predisposition (1-3)	Apolipoprotein-1 (APOL1) genetic variants

[1] TTP – Thrombotic Thrombocytopenia Purpura, HUS – Hemolytic Uremic Syndrome
[2] ART – Antiretroviral Therapy

Renal replacement therapy (RRT) for patients with ESKD comprises two modalities – kidney transplantation and chronic dialysis therapy which can be either hemodialysis or peritoneal dialysis.

Chronic dialysis therapy is expensive for multiple reasons. At a health systems level, provision of chronic dialysis services require highly trained medical professionals, expensive equipment that needs maintenance, high volume consumables, water purification systems (for hemodialysis) and a dedicated space for dialysis that has access to in-hospital services. [4] For the individual with ESKD, chronic dialysis requires regular monitoring of critical indices with blood tests, expensive pharmacotherapeutics such as parenteral iron and erythropoietin and creation and ongoing patency of access for dialysis, either with a peritoneal catheter or vascular access for hemodialysis. This adds substantial cost for health care providers (whether state or private) and, when not funded, can be passed onto individuals as "out of pocket" expenses. These dialysis-related expenses occur in addition to the costs of treating additional comorbidities such as hypertension, diabetes and HIV infection. [5]

Limited hemodialysis slots are available for patients with ESKD. (6) In South Africa, access to RRT is disparate, with 189 slots for renal dialysis per million population overall, but only 71.9 per million population available to the public sector. [6] There is currently no national policy regulating access to RRT in South Africa. A recent audit done in the Western Cape revealed that, of all the patients receiving dialysis, only 10% were PLWH. [7] In view of these limitations, kidney transplantation is an attractive option. Kidney transplantation is a curative therapy which prolongs life in patients with ESKD and is more cost-effective even in complicated cases with high levels of sensitisation. [8] [9]

Kidney transplantation in PLWH has shown improved overall survival outcomes when compared to PLWH on chronic hemodialysis. [10] Morbidity and mortality data also suggest that outcomes after renal transplantation are similar in HIV-infected and uninfected patients. [11, 12] HIV infection was previously considered a contra-indication for both chronic hemodialysis and renal transplantation in South Africa but policy has been revised. [13] This is in line with regulations internationally including the 2013 United States HIV Organ Policy Equity (HOPE) Act. [14] This law also authorized the use of HIV-infected organs for transplantation in PLWH. Outcomes in PLWH patients undergoing kidney transplantation in South Africa are equivalent to those seen in other studies for both HIV-infected and uninfected donor pools. Some centres in South Africa have begun utilising organs from HIV infected deceased donors with 100% 1 year graft survival. [13]

The Johannesburg kidney transplant programme is the largest national programme and transplants approximately 20-25 deceased donor kidneys per year. Listing of PLWH patients commenced in (2014). The current guidelines for eligibility for deceased-donor kidney transplantation in an HIV-infected individual in the Johannesburg transplant programme include: stable ART therapy with good adherence for the past 6 months, absence of acquired immunodeficiency syndrome (AIDS)-defining illnesses, CD4+ T cell counts of >200 cells/µl for 6 months and undetectable viral load for more than 6 months. [15]

CD4+ T cell count is an important risk predictor in patients undergoing transplantation. Patients with absolute CD4+ T cell count of <200 cells/µl are at an increased risk for opportunistic infections, have a higher post-transplant rejection rate and present with delayed CD4+ T cell count recovery after the procedure. [16] Although HIV infection is the primary driver of the reduced CD4+ T cell count in PLWH, other factors may also impact on

the peri-transplant immune status of patients including the use of chronic hemodialysis. Previous studies, examining the impact of hemodialysis on leucocyte counts and leucocyte subsets, have been performed in the past on HIV-uninfected cohorts. The findings of these studies are contradictory. Generally, they showed consistently decreased levels of CD3+, CD4+ and CD8+ T cells however these measurements were taken at various intervals between hemodialysis and not necessarily immediately post-dialysis. [11] These studies postulated that direct contact between lymphocytes and dialyser membranes can result in activation of lymphocytes with subsequent apoptosis. [11, 12]

A concern therefore exists that chronic haemodialysis could reduce CD4+ T cell count especially in PLWH and this would impact their eligibility for the deceased donor list. The aim of this study was to measure immediate and ongoing T cell counts and T cell subsets to evaluate the impact of dialysis on T cell recovery in PLWH with ESKD receiving chronic hemodialysis.

Methods

Ethical approval was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (M170858).The study participants were recruited from Helen Joseph Hospital, Chris Hani Baragwanath hospital, Charlotte Maxeke Johannesburg Academic Hospital and Donald Gordon Medical Centre.

Informed consent was obtained from the study participants and the controls. Only patients who refused or were otherwise unable to give consent were excluded.

Study participants

The study participants were HIV-infected adults with ESKD receiving chronic hemodialysis (three sessions a week, each lasting ~4 hours), irrespective of their treatment regimens, immunological or virological parameters.

Demographic and clinical information was collected, including the presence of co-morbid diseases, drug history, social habits, the presence of chronic infections, the underlying cause for ESKD and ART regime. All available CD4+ T cell counts and HIV viral loads were documented.

Study controls

The study participants were matched to HIV-uninfected patients with ESKD receiving chronic haemodialysis. The control group was matched to the HIV-infected group for age, sex and body mass index.

Sample collection

Vascular access was established immediately prior to haemodialysis. Peripheral whole blood samples were collected with a needle and a syringe and placed in a 4.5 ml EDTA tube. Haemodialysis was initiated and continued for four hours. A second whole blood sample was taken within 10 minutes after the end of dialysis ended in a similar manner than the pre-dialysis sample. The samples were transported at room temperature to the laboratory within 24 hours of collection.

Laboratory performance of the T cell subsets

All CD4+ T cell counts were analysed by flow cytometry. Briefly, 100 µL of whole blood was incubated for 10 minutes in an automated T-Q-Prep machine (Beckman Coulter, Florida Inc.) with 5 µL Cytostat tetraCHROMETM CD45 (FITC)/CD4(RD1)/CD8(ECD)/CD3 (PC-5) monoclonal antibody (Beckman Coulter, Ireland Inc). During the incubation period a stabilizer, lysing agent and fixative were added. 100µL of Flow CountTM Beads (Beckman Coulter) were then added to the lysate and analysed on a Beckman-Coulter FC500-MPL flow cytometer on a 4-colour T-cell protocol. Absolute T-cell numbers were then calculated using the total White Cell Count (WCC), the percentage (%) lymphocytes and the percentage (%) CD3 or CD4 or CD8 cells and expressed as both an absolute number (cells/ µL) and as a percentage of the white cell count. [17] The CD4+ T cell count was compared with the laboratory-determined reference range. In four study participants only CD4+ T cell counts could be performed.

Longitudinal data analysis

All possible CD4+ T cell counts and HIV viral loads available for the study participants were documented.

Statistical analysis

All continuous variables (including the CD4+ T cell count) were expressed as a median and interquartile range and a mean and a standard distribution. A normality test (D'Agostino & Pearson normality test) was applied to the data set. Comparisons between pre- and post-dialysis parameters were performed using a paired student's T-test.

The trend analysis of the absolute CD4 counts, the % of CD4 cells and the viral loads were analysed using a simple linear regression. All the statistical data were analysed using GraphPad Prism 7.05. A *p* value of <0.05 was considered significant for these analyses.

Results

Socio-demographic data of the study participants

A total of 17 participants and 17 age, sex and BMI matched controls were included in this study. All the study participants had ESKD and were receiving RRT by means of chronic hemodialysis (three sessions per week, each session lasting ~4 hours).

Renal biopsies had not been performed in most participants and in the majority of cases; the cause of renal failure was inferred from the patient medical records. The most common cause for ESKD was stated as hypertension (82%). Most of the study participants had

uncontrolled hypertension. Two patients had (renal biopsy confirmed) HIVAN (12%) and one patient had renal failure due to ethylene glycol overdose (0.05%).

All HIV-infected patients were treated with first line ART regime at doses adjusted for kidney failure. It is unclear if these patients were already on ART prior to hemodialysis commencement. All HIV-infected participants had received a GeneXpert (Cepheid, Sunnyvale) test for *Mycobacterium tuberculosis* prior to commencement of hemodialysis. Only a single patient had Hepatitis B Virus co-infection. The sociodemographic details are summarized in table 2.

Table 2: Socio-demographic and categorical variables of the study participants

	Study participants
Age in years (mean, ±SD)	38 (±4.8)
BMI[3] (mean, ±SD)	25 (±5.3)
Duration of ART[4] **treatment years** (mean, ±SD)	5 (±1.8)
Sex	
Male (n, %)	9 (53)
Female (n, %)	8 (47)
Co-morbidities	
Hypertension (n, %)	17 (100)
Diabetes Mellitus (n, %)	0 (0)
Social history	
Reported smoking (n, %)	0 (0)
Reported alcohol use (n, %)	0 (0)
Reported recreational drug use (n, %)	0 (0)
Chronic infections	

[3] BMI – Body Mass Index
[4] ART – Antiretroviral Therapy

Mycobacterium Tuberculosis (n, %)	0 (0)
Hepatitis B (n, %)	1 (0.1)
Cause for renal failure	
Hypertension (n, %)	14 (82)
HIVAN[5] (n, %)	2 (12)
Other (n, %)	1 (0.05)

[55] HIVAN - HIV-associated nephropathy

T cell subsets of the study participants

Leucocyte count and T cell subsets were measured immediately before and after a single session of hemodialysis for the study participants. These results are summarized in table 3.

All of the study participants' total leukocyte counts, absolute CD3+ T cell counts and the absolute CD8+ T cell counts were within the normal laboratory reference range for adults. The pre-dialysis absolute CD4+ T cell count was lower than the normal laboratory reference range in eight HIV-infected patients (47%), and two patients had an absolute CD4+ T cell count less than 200 cells/μL. The post-dialysis absolute CD4+ T cell counts were lower than the normal reference range in five of the HIV-infected patients (29%). Only one patient presented with an absolute CD4+ T cell count of less than 200 cells/μL.

Table 3. Measured parameters of the study participants.

	WCC[6] (x 10^9/L)		% of CD3+ cells lymphocytes		Absolute CD3 count (cells/µL)		% of CD4+Tcells		Absolute CD4 count (cells/µL)		% of CD8+ T cells		Absolute CD8 cells (cells/µL)	
	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis	Pre-dialysis	Post-dialysis
1	5,24	4,43	78.4	83.5	575	652	24,7	29,6	181	231	50,4	51,1	370	399
2	2,74	2,85	82.3	83.6	546	777	31,5	37,2	209	346	49,6	44,9	330	417
3	5,11	3,6	73.3	75.6	1174	720	24,1	29,8	387	284	48,4	44,5	778	424
4	5,38	3,96	84.4	86.5	740	813	39,3	40	345	376	44,2	43,7	388	411
5	6,52	5,86	71.5	75.7	609	584	38	47,4	324	366	31,4	27,3	267	211
6	6,97	6,47	73.1	79.5	1009	690	10	15,2	139	132	57,5	58,8	794	511
7	4,12	6,88	78.5	78.0	777	1400	40,8	43,7	404	785	34,8	31,9	345	574
***8**	2,94	2,95	-	-	-	-	29,8	32,7	227	281	-	-	-	-
***9**	3,95	3,94	-	-	-	-	25	29,6	260	328	-	-	-	-
***10**	3,25	3,8	-	-	-	-	38,4	44,2	414	521	-	-	-	-
***11**	5,3	6,07	-	-	-	-	28,2	36,5	222	330	-	-	-	-
12	6,88	6,63	78.8	82.9	1905	1734	38,4	46,6	929	975	37,2	33,3	901	697
13	3,65	4,2	82.1	83.5	834	980	27	36,4	274	428	51,8	43,9	526	515
14	5,86	5,17	79.1	87.5	1576	1270	44,5	54	888	784	27	27,1	538	393
15	3,63	3,07	75.2	75.5	766	765	33,75	34,4	349	357	40,3	39,4	416	408
16	5,62	5,26	73.4	77.3	770	870	44,6	50,4	483	564	27,3	26,9	287	301
17	7,11	7,03	78.3	78.6	1390	1294	29,8	30	538	544	46,7	43,2	843	750
Mean	**4,69**	**4,83**	**78.35**	**79.05**	**777**	**813**	**32,23**	**37,51**	**386,6**	**448,9**	**42,05**	**39,69**	**521,8**	**462,4**
	***P* <.61**		***P* <.14**		***P* <.90**		***P* <.001**		***P* <.03**		**P <.008**		***P* <.20**	

[6] WCC – White Cell Count

* CD8% and absolute count not collected.

There was no statistically significant change in the total leukocytes count, (t=0.5178, *P*<.61), the CD3+ cells as a % of the lymphocyte count (t=1.609, *P*<.1359) or the absolute CD3+ T cell count (t=0.122, *P*<.90) after hemodialysis. A statistically significant increase was noted in the post-dialysis CD4+ T cells as a % of lymphocytes (t = 7.106, *P*<0.001) as well as the absolute CD4+ T cell count counts (t = 2.371, *P*<.03). A statistically significant decrease in the post-dialysis % of the CD8+ T cells (t=3.212, *P*<.008) (figure 1).

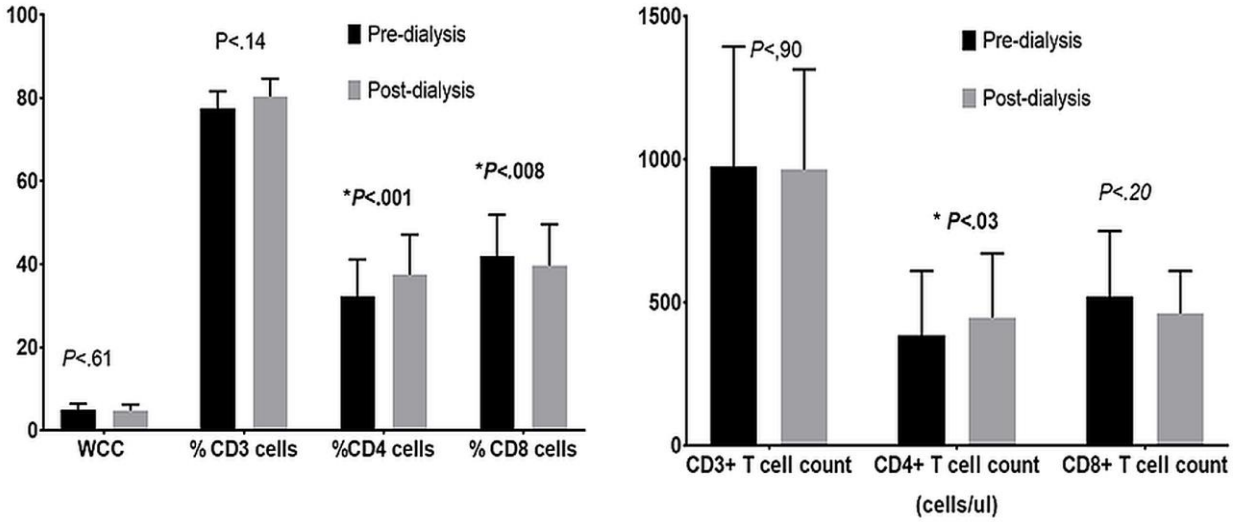

Figure 1. Measured parameters of study participants pre- and post-dialysis. Absolute CD4+ T cell count and CD4+T cell counts showed a significant increase immediately post-dialysis

T cell subset of the study controls

T cell subsets (% and absolute) for the HIV-uninfected controls were within the normal adult reference ranges pre- and post-hemodialysis. The % of CD4+ T cells was the only immunological parameter to show a statistically significant increase after hemodialysis (t=4.195, *P*<.001). The other hematological parameters revealed no statistically significant change.

Longitudinal trends of the percentage CD4+ T cells, absolute T cell count and HIV viral loads of the study participants

Following a cross-sectional analysis, the longitudinal trends for the % of CD4+ T cells, the absolute CD4+ T cell counts and the HIV viral loads were analyzed for each patient. The initial available CD4+ T cell count was taken as point zero. Although the exact date on which hemodialysis was started for each patient is not certain, it is known that point zero was obtained prior to hemodialysis initiation.

Five HIV-infected study participants (patients 1, 4, 7, 10 and 11) showed a statistically significant longitudinal increase in the CD4+ T cells as a % of white cell count and 8 showed a trend towards increased CD4+ T cell % which was not significant. Only 1 patient had a trend towards a reduced CD4+ T cell count which was statistically significant. (Figure 2)

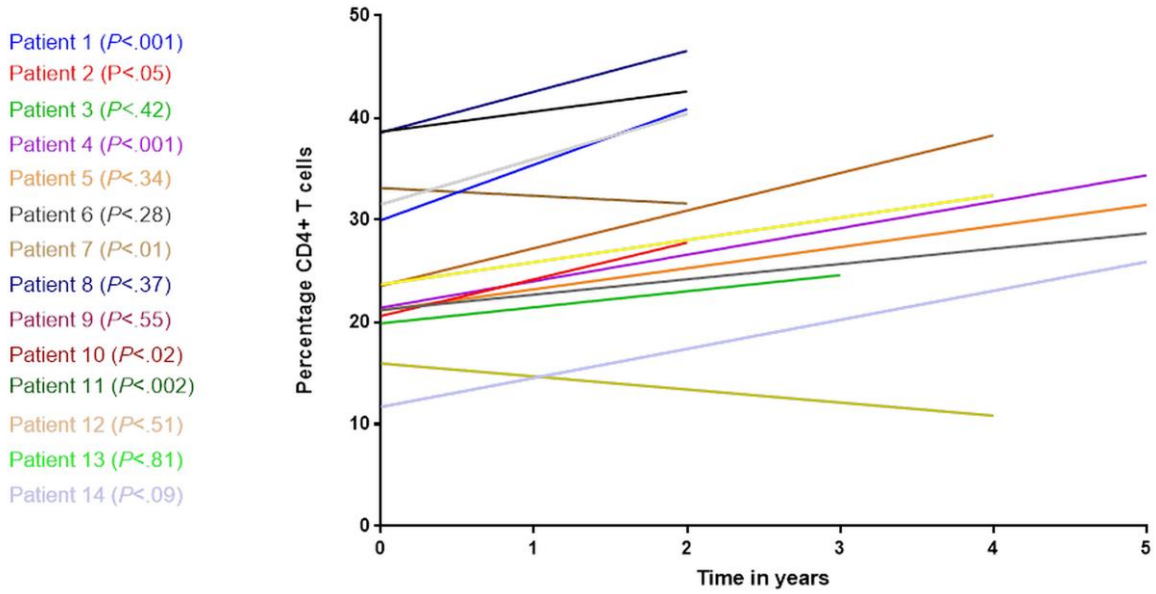

Figure 2: Linear regressions of the percentage of CD4+ T cells.

The linear regression analyses for the absolute CD4+ T cell counts for the study participants revealed only a single statistically significant result. Patient 11 showed a statistically significant decrease in the absolute CD4+ T cell counts. (Figure 3).

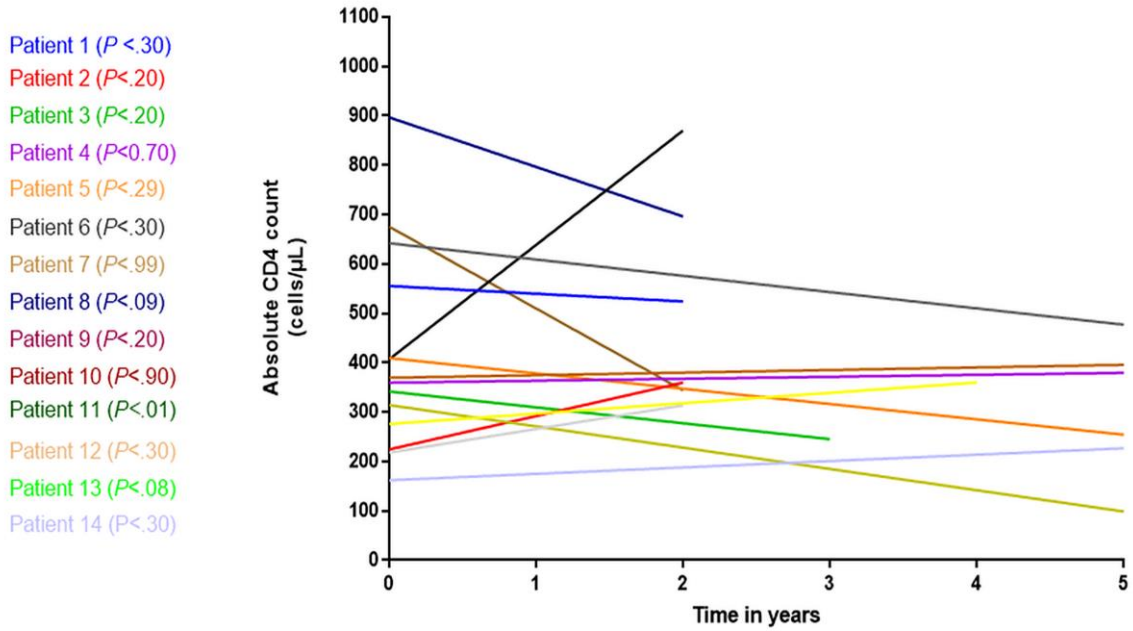

Figure 3: Linear regression of absolute CD4+ T cell counts

Virological suppression is a pre-requisite for eligibility for deceased donor kidney transplantation. Although all HIV-infected patients were receiving standard first-line ART, only three study participants showed virological suppression below the level of detectability. The cross-sectional median viral load was 44 500 copies/ml (±41944,64). In patients for whom longitudinal data were available, most patients displayed a stable viral load (n=13). Only one patient had a statistically significant increase in the HIV viral load.

Discussion

Kidney transplantation is a cost-effective and curative strategy in patients with ESKD irrespective of whether they have HIV infection or not. Eligibility criteria could, however, limit access to this treatment particularly if these could be impacted by RRT. A CD4+ T cell count above 200 cells/ul is a pre-requisite for eligibility for deceased donor kidney transplantation in the Johannesburg transplant program. This study investigated whether alternative forms of RRT, specifically hemodialysis, materially reduced the CD4+ T cell count.

CD4+ T cell count as a % of lymphocytes and as an absolute number increased immediately following hemodialysis in HIV-infected participants. These findings contradict previously published data which suggest that absolute CD4+ T cell counts decline immediately post-dialysis. [11] This represented a true increase rather than the effect of hemoconcentration post-dialysis since the absolute leukocyte count and the absolute CD3+T cell count showed no significant change post-dialysis. None of the study participants or controls had a CD3+ T cell lymphopenia or a decreased CD8+ T cell count before dialysis. The HIV-infected study participants had significantly lower CD4+ T cell counts prior to dialysis than uninfected controls.

The % of CD8+ T cells and absolute CD8+ T cell count did show a significant decrease post-hemodialysis in the HIV-infected study participants. Previous studies in HIV-uninfected patients with ESKD receiving hemodialysis, revealed lower levels of absolute CD8+ T cells when compared to normal controls. [18-20] The CD8+ T cell count decrease is postulated to be caused by activation of these cells by the dialyzer membrane with subsequent apoptosis of these cells. [18, 21]

We went on to assess the longitudinal trends of % of CD4+ T cells, absolute CD4+ T cells and the HIV viral loads based on retrospective laboratory data for fourteen of our study participants. The majority of our population showed a stable (n=8) or increasing CD4+ T cell count over time (n=5) above 200 cells/µL. This is the minimum absolute CD4+ T cell count required listing for deceased donor transplantation although ongoing investigations are being conducted to establish the optimal absolute CD4+ T cell count for the best possible outcome. It appears that an absolute CD4+ T cell count of 200 cells/µL may be inadequate to protect against adverse outcomes including post-transplant opportunistic infections. [16] A similar study done in our setting found an annual increase in the absolute CD4+ T cell counts in PLWH with end ESKD on chronic hemodialysis. [22]

The majority of the study participants were not virologically suppressed and the frequency of HIV viral load testing performed varied amongst the different centers treating these patients. The current national HIV treatment guidelines state that the immunological (CD4+ T cell count) and virological (HIV viral load) parameters should be measured at six-monthly intervals in PLWH and it is uncertain if the infrequent viral load testing is contributing to the virological state of the study participants. [23] The majority of our patients were not virologically suppressed which is also a criterion for deceased donor kidney transplantation eligibility. This is not an uncommon finding in PLWH with ESKD on chronic hemodialysis. Studies assessing the longitudinal HIV viral loads in PLWH with ESKD on chronic renal dialysis found approximately half of their cohort of patients did not have a suppressed HIV viral load. [22, 24] Possible reasons include hemodialysis, itself, which may lead to an increase in HIV replication because of the release of specific cytokines as well as the use of certain dialysis membranes used during the hemodialysis procedure. [25, 26] Other possible causes include inexperience with prescribing ART (suboptimal dosing due to the renal failure),

infrequent consultations with Infectious Diseases Specialists, patient compliance and ART-timing (before or after hemodialysis) which could influence drug concentrations. [22]

This study has some limitations. The number of PLWH with ESKD currently receiving chronic hemodialysis in 4 different academic centers in Johannesburg, are small. It is likely that the small number reflects the strict qualification criteria for dialysis and the limited dialysis slots available. In addition, data on longitudinal CD4+ T cell counts and viral loads were not always available and the timing of testing was inconsistent. It was not possible in this small study to perform ART drug monitoring to ensure that the absence of virological suppression did not reflect lack of compliance.

This study failed to show any effect of hemodialysis on CD4+ T cell count, however. Unexpectedly, the absolute CD4+ T cell count increases immediately post-dialysis suggesting that immunologic recovery is not impeded by treatment of the underlying ESKD. This appears in this study to be a prolonged rise with 13 of 14 of the patients assayed showing an increase in CD4+ T cell percentages. Further studies are required to ascertain possible reasons for a rise, how long this rise is sustained and whether these CD4+ T cells are functional. Of concern, the patients in this study failed to show virological suppression and since this is a key driver of disease progression and complications including non-communicable diseases, this requires urgent investigation.

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Appendices

Appendix A: Approved research protocol

Factors influencing CD4+ T-cell counts in people living with HIV with end-stage renal disease

Dr. Melanie Pretorius

Department of Haematology and Molecular Medicine

National Health Laboratory Service and the University of the Witwatersrand

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1. List of abbreviations

AIDS – Acquired Immunodeficiency Syndrome

ART – Antiretroviral therapy

BMI – Body mass index

eGFR – estimated Glomerular Filtration Rate

ESRD – End Stage Renal Disease

HIV – Human Immunodeficiency Virus

HIVAN – HIV-associated nephropathy

HIVICD – HIV-associated immune complex disease

NHLS – National Health Laboratory Service

PLG - PanLeucogating

µl – Microlitre

WHO – World Health Organisation

2. Title

Factors affecting CD4+ T-cell counts in HIV-infected patients with end-stage renal disease.

3. Protocol summary

This study will be a prospective laboratory-based study to assess the effect of dialysis and co-morbidities on the CD4+ T cell counts in HIV-infected patients with end-stage renal disease. Currently, the guideline for a renal transplant in an HIV-infected patient with chronic renal disease requires a CD4+ T-cell count of at least 200 cells/microlitre (µl). It has been noted that a small number of these patients reach a CD4+ T-cell count of 200 cells/µl and the role dialysis plays has not been investigated. A blood sample will be taken from the study participants prior to dialysis and immediately thereafter. The CD4+ T-cell count of the study participants will be measured on these blood samples and the trend of the CD4+ T-cell count of the study participants will be assessed. The CD4+ T-cell count trend of the study participants will be compared to age and sex matched HIV-uninfected controls. The impact of certain clinical factors on the study participants' CD4+ T-cell counts will be evaluated. Study participants will be used from Charlotte Maxeke Johannesburg Academic Hospital, Chris Hani Baragwaneth Academic Hospital and Helen Joseph Hospital.

4. Introduction

Renal disease in HIV-infected patients

The prevalence of HIV associated renal disease varies in Africa, depending on the population studied and the criteria used for chronic kidney disease. In South Africa the prevalence is about 6%(11).As many as 83% of HIV-infected South African patients, who are not on treatment, will develop renal failure(27).Approximately 10% of HIV-infected patients on

antiretroviral treatment will develop end-stage renal failure(13).Based on the number of patients diagnosed with HIV in South Africa, it is estimated that ~550 000 patients will need renal replacement therapy(13).

Ageing in HIV-infected patients increases the risk of HIV-associated chronic renal diseases. These include classical HIV-associated nephropathy (HIVAN), HIV-associated immune complex disease (HIVICD), HIV-associated thrombotic microangiopathy (thrombotic thrombocytopenic purpura and haemolytic uremic syndrome) and comorbid diseases (Hepatitis C infection, diabetes mellitus and hypertension)(28).

Of these diseases, HIVAN is the best described. Clinically HIVAN presents with heavy proteinuria, estimated glomerular filtration rate (eGFR) and echogenic kidneys on ultrasound(29).These patients typically present late with advanced kidney failure due to lack of screening for renal dysfunction and the absence of overt symptoms(30).HIVAN usually occurs in states of advanced immunosuppression and in young Africans. In patients of African descent, the risk of developing HIVAN is increased 18 fold(31). Histological features include focal segmental sclerosis, microcystically dilated tubules and tubulointerstitial inflammation(2).HIVAN has been classified as HIV stage 4 disease by the World Health Organisation (WHO) and this diagnosis warrants the initiation of antiretroviral therapy irrespective of CD4+ T-cell count(28).

HIVICD is a diverse group of diseases. They are more prevalent in European and Asian populations withthe prevalence in Africa ranging from 17% to 40%. They include immune complex glomerular disease, IgA nephropathy, mesangial proliferative nephropathy, membranoproliferative nephropathy, exudative proliferative nephropathy and lupus-like proliferative glomerulonephritis(32).

Combined antiretroviral therapies (ART) are also associated with kidney disease e.g. proximal tubular nephropathy which is seen with treatment with tenofovir(33)and nephrolithiasis which is associated with ritonavir use(34). This renal damage may be irreversible even after the drug is stopped(35) and the risk of renal damage may be increased by other clinical factors including patient age, comorbidities and associated treatment(36).

Renal replacement therapy in HIV infected individuals.

Renal dialysis is the process by which waste and excess water in blood is removed artificially by a haemodialysis machine. The frequency of haemodialysis is usually three times per week(37). Peritoneal dialysis is a method whereby a cleaning solution, dialysate, absorbs waste and fluid from the body, using the peritoneum as a filter. Both these modalities have various side effects. These include cardiovascular effects, infections, dialysis-access related issues, malnutrition, lethargy, itchiness, dizziness, gastro-intestinal disturbances, depression and other psychological problem(38). The loss of CD4+ T-cells is not a recognisable side effect of dialysis. Renal transplantation improves the morbidity and mortality in HIV uninfected and HIV infected patients (3).

The current South African guidelines for renal transplantation, state that HIV-infected patients with end-stage renal disease are only eligible for transplantation if they:

- Demonstrate stability of ART therapy with good adherence for the past 6 months.
- Absence of acquired immunodeficiency syndrome (AIDS)-defining illnesses
- Maintain CD4+ T cell counts of >200 cells/µl for 6 months
- Show an undetectable viral load for more than 6 months(37).

The requirement for a CD4+T cell count above 200 cells/µlis to minimize the risk of opportunistic infections which could either prejudice graft survival or become exacerbated by post-transplantation immunosuppression. Prophylaxis is available for some of these infections but is not available for others such as Human Herpes Virus-8 and Creutzfeld-Jacob disease, which are associated with CD4+ T-cell counts less than 200 cells/µl)(39).

International studies predict that only 20% of HIV infected patients with end-stage renal disease reach the waiting list for renal transplantation. The main reasons for this were a low CD4+ T-cell count and a history of substance abuse(40).Certain factors which may lower the CD4+ T-cell counts in HIV infected patients include age of the patient, the use of illicit drugs, use of tobacco, hospital treatment, changing of doctors and the use of antiretroviral treatment(41). Further studies have also demonstrated that overweight patients (BMI 25-2.9 kg/m^2) have the best CD4+ T-cell response to ART. In contrast underweight patients (BMI <20 kg/m^2) and obese patients (BMI >30 kg/m^2) were associated with lower a CD4+ T-cell response to ART(42).

In South Africa, studies have been undertaken to assess the impact of renal transplantation among HIV infected patients. Transplantation is more cost effective than chronic renal dialysis and dialysis slots, especially in the public sector are limited(12).

In view of the increasing incidence of the spectrum of chronic renal disease in HIV infected patients, there will be an increased burden of patients needing renal replacement therapy(13). Previous studies suggest that, only a few patients reach the desired CD4+ T-cell count to achieve the waiting list for a renal transplant. Anecdotal evidence suggests that this may be related to the renal replacement therapy although there are no data to

support this hypothesis. This study is a pilot project to evaluate whether dialysis has any demonstrable effect on renal transplant patients.

5. Investigators

The principle investigator will be Dr Melanie Pretorius (junior registrar in the Department of Molecular Medicine and Haematology (MM&H) of the University of Witwatersrand, National Health Laboratory Services (NHLS)).

The supervisors are Dr Elizabeth Mayne (consultant haematopathologist in the department of MM&H, NHLS), Dr Denise Lawrie (senior scientist, CD4 laboratory) and Dr Estee L Benade (consultant haematopathologist in the department of MM&H, NHLS).

6. Hypothesis, aim and objectives

6.1 Hypothesis

Dialysis plays no significant role in preventing patients with HIV infection and concomitant end-stage renal disease from achieving a CD4+ T cell count of 200 cells/µl.

6.2 Aim

To assess the trend of the CD4+ T-cell count of HIV infection with end-stage renal disease receiving dialysis.

6.3 Objectives

To assess the CD4+ T-cell count of these patients pre- and post-dialysis using the Pan Leucogated (PLG) CD4 T-cell enumeration technique.

To determine epidemiological and disease factors which could impact on CD4+ T cell count and fitness for transplantation within a cohort of HIV-infected patients with end-stage renal disease.

To assess the impact of these clinical factors on the CD4+ T-cell count of the study participants. (Refer to appendix A for data collection sheet).

7. Methods

7.1 Sample material

The study participants will be recruited from Helen Joseph Hospital, Chris Hani Baragwaneth hospital and Charlotte Maxeke Johannesburg Academic Hospital. Informed consent and pertinent clinical information will be obtained from the study participants. Two patient populations will be studied:

1. HIV-infected patients with end-stage renal disease. Based on preliminary data, we have identified that approximately 50 patients attending dialysis are HIV-infected.
2. HIV-uninfected controls. These will be patients with end-stage renal disease without HIV-infection. This control group will be matched to the HIV-infected group for age, gender and body mass index.

Peripheral blood samples will be taken in an EDTA blood tube prior to dialysis and immediately thereafter. The enumeration of the CD4+ T cell count will be performed using the PanLeucogating (PLG) method. This method involves enumeration of the total white cell count and then the CD4+ T-cells as a percentage of this population. This enumeration will be performed for each study participant and control pre-and post-dialysis. If any additional

readings for the CD4+ T-cell count are available on the laboratory information system for consented patients, these will be recorded with their respective time points.

The following data will be obtained from each participant:

1. Age
2. Gender
3. Co-morbid diseases which could impact renal function and their control:
    1. Diabetes mellitus. Control will be defined by an $HbA1_c$ of below 7%(43).
    2. Hypertension. Control is defined as a blood pressure below 140/90 mm Hg(44).
4. Chronic infections: These include any chronic infections other than HIV which are listed in the patient's clinical file. Specifically, it will include Mycobacterium spp. infections, chronic hepatitis and gamma-herpesviridae infections
5. Antiretroviral Therapy : current antiretroviral regimen, duration and control (as assessed by HIV viral load)
6. Social habits:
    1. Tobacco use – measured as pack years (number of packs smoked per day multiplied by number of years as a smoker) (45).
    2. Alcohol – measured as alcohol units per week. One unit of alcohol is defined as 25ml spirits, a glass of wine or a 340ml bottle of beer (46). (Refer to Appendix A for data collection sheet).

7. Body mass index – this is defined as the weight divided by the height^2 and has been shown to impact CD4+ T-cell count. This will be expressed as a figure. The weight will be assessed post-dialysis (referred to as a dry weight) (42).
8. The laboratory number for each study participant sample, to keep track of the CD4+ T-cell count result of each study participant.
9. The date and time of the sample collection and the date and time of haemodialysis.
10. The result of the study participant's CD4+ T-cell count prior to dialysis.
11. The result of the study participant's CD4+ T-cell count post dialysis.

7.2 Sample analysis

7.2.1 Principle of PanLeucogating (PLG) CD4+ T-cell count enumeration

CD4+ T-cell count enumeration by this method involves two measurements. These include the measurement of the total white blood cell count and then identifying the CD4+ T-cells within the total white cell population. The total white cell count is measured using single Bead flow cytometry counting. The CD4+ T-cell percentage is then measured on the same whole blood sample by flow cytometric immunophenotyping. Anti-CD4 and Anti-CD45 monoclonal antibodies (Beckman-Coulter, California USA) are added to the sample. These monoclonal antibodies are in contained in the Beckman Coulter Flow Care™ PLG CD4 kit. The cells that have bound the labelled antibody are identified by the flow cytometric analyser by means of the specific fluorescence emitted by the specific fluorochrome attached either to the CD4 or CD45 antigen. The CD4+ T-cells are further distinguished from the rest of the white blood cells on the basis of their complexity and their expression of CD45 and CD4(17).

7.2.2 Specimen

A peripheral (whole) blood sample will be collected in a 5ml EDTA blood tube and kept at room temperature. The CD4+ T-cell count will be analysed within 48 hours after sample collection.

7.2.3 Handling conditions of the specimen

These samples will be promptly transported to the laboratory to ensure that they are analysed within the allocated time. Once the whole blood sample reaches the laboratory, the samples will be prepared and stored at room temperature. The prepared haemolysates will be analysed at room temperature within 6 hours after preparation. If for any reason the analysis can only be performed after 6 hours of preparation, they will be kept covered at 2-8°C.

7.2.4 The method of performing a CD4+ T-cell count

The whole blood samples will be placed on the blood mixer to ensure adequate mixing of the blood prior to pipetting of the whole blood.

For each whole blood sample received, a separate tube will be prepared. 10µl of Flow Care PLG CD4 monoclonal antibodies will be pipetted into the bottom of each sample tube. 100µl of the mixed, whole blood sample will be added to the labelled tube containing the monoclonal antibodies and the tube will be vortexed for 2 minutes.

The sample will be incubated in the dark for 10-15 minutes at 15-25 C. After incubation the samples will be loaded into the T-Q prep carousel. 100µl of Flow count beads will be pipetted into the haemolysate. The haemolysate samples will be loaded within 2 hours onto the flow cytometry XL-MC analyser and analysed using the CDARV protocol(17).

7.2.5 Reporting of the CD4+ T-cell count

The flow cytometry XL-MCL analyser measures the white cell count, the CD4% of the lymphocytes and the CD4% of the total white cells (kindly refer to appendix B for an example of the results sheet). The absolute CD4 count is calculated using the following formula:

White blood cell count x %lymphocytes x %CD4 of lymphocytes / 10 = CD 4+ Tcell number

7.3 Sample size

Sample size will be determined by the number of HIV-infected patients presenting for dialysis at the study sites over the trial period. Ideally, this will include 50 HIV-infected patients and 20 controls. The control group will include HIV-uninfected patients undergoing dialysis for end stage renal disease. The control group will be age-, gender- and BMI-matched to the study participants.

A post-hoc analysis will be performed after the results of 10 HIV-infected and 10 HIV-uninfected individuals are obtained to estimate the sample size required to avoid a type 1 error. If necessary, the sample size will be increased by recruiting HIV-infected patients with end-stage renal disease from private facilities including Donald Gordon Medical Centre.

7.4 Inclusion and exclusion criteria

This prospective study will include all HIV-infected patients with end-stage renal disease on dialysis and 20 age-, sex- and BMI matched control participants. Participants that do not give consent will be excluded from this study. Samples collected in heparin blood tubes, that are haemolysed or frozen and that reach the lab after 48 hours will be rejected.

8. Data analysis

All continuous variables (including the CD4+ T cell count) will be expressed using descriptive statistics. If the data are normally distributed, they will be expressed as a mean and standard deviation. A comparison in this case between pre- and post-dialysis CD4+ T cell counts will be performed using a paired student's T-test. If the data are not normally distributed, they will be expressed as a median and interquartile range and a difference between the pre- and post-dialysis CD4+ T cell count will be analysed using a Wilcoxon sign-rank test. To ensure inter-assay reproducibility, each CD4+ T cell test will be performed in duplicate although the reported coefficient of variation of the test and analyser is consistently below 5%. A table on uncertainty of measurement for the CD4+ T cell count at the CMJAH CD4 flow cytometry laboratory is included as appendix D. And this information will be used in determining if the change in CD4 count is statistically valid.

The impact of categorical variables including the presence or absence of infections, co-morbid disease and disease control and treatment regimens on CD4+ T-cell count will be assessed using a logistic regression.

A p value of <0.05 will be considered significant for these analyses.

9. Ethics

Permission to collect samples has been submitted to the 3 government hospitals. When this approval is obtained, a formal submission will be made to the Human Research Ethics Committee of the University of the Witwatersrand.

10. Study period

Prospective data collection for this study will commence once ethics and postgraduate committee approval has been obtained and will continue for 1 year (refer to Appendix D for Gant chart).

11. Budget and funding

Additional funding will be sought from the University of the Witwatersrand Faculty of Health Sciences as individual grants from the Faculty Research Committee. Additional funding will be provided, where necessary, by Dr Mayne through her grant funding mechanisms. Table two shows a detailed budget.

Table 2: Proposed budget

	Cost per test	Cost for participants and controls (140 tests)
Equipment cost: Including analysers, mixers, fridge, pipettes, bio-hazard safety cabinet, computers, air conditioner	R12.27	R1718.80
Reagents and consumables cost: Including gloves, sharps containers, printer paper, printer cartridges and PLG-reagents	R42.94	R6011.60
Total cost	R55.21	R7729.40

12. Limitations

With the prospective nature of this study, there is a possibility of inadequate sample size for statistical significance.

CD4+ T-cell counts will be measured for each participant at only 2 time points (in duplicate). This may not provide the most accurate trend of the CD4+ T-cell count post dialysis. Further studies measuring more than two serial CD4+ T cell counts may be needed to ascertain the most accurate trend of the CD4+ T-cell count post-dialysis.

Only three centres are listed in this protocol, however should the sample size not be reached, other centres may be included in this study.

Appendix A: Data collection sheet

Laboratory number	
Age	
Gender	
ART regimen	
Duration of antiretroviral therapy	
Co-morbidities	
Diabetes	Yes or No
Treatment	
Control (HBA1C <7%)	Yes or No
Hypertension	
Treatment	
Control (blood pressure <140\90)	Yes or No
Chronic infections other than HIV **Specify chronic infection**	Yes or No
Treatment	
Smoking Number of pack years	Yes or No
Alcohol use Number of units per week	Yes or No
Cause of renal failure	
CD4+ T cell count pre-dialysis	
CD4+ T cell count post-dialysis	
Body mass index (dry)	

Appendix B: Results sheet

FLOW CYTOMETRY XL-MCL4

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

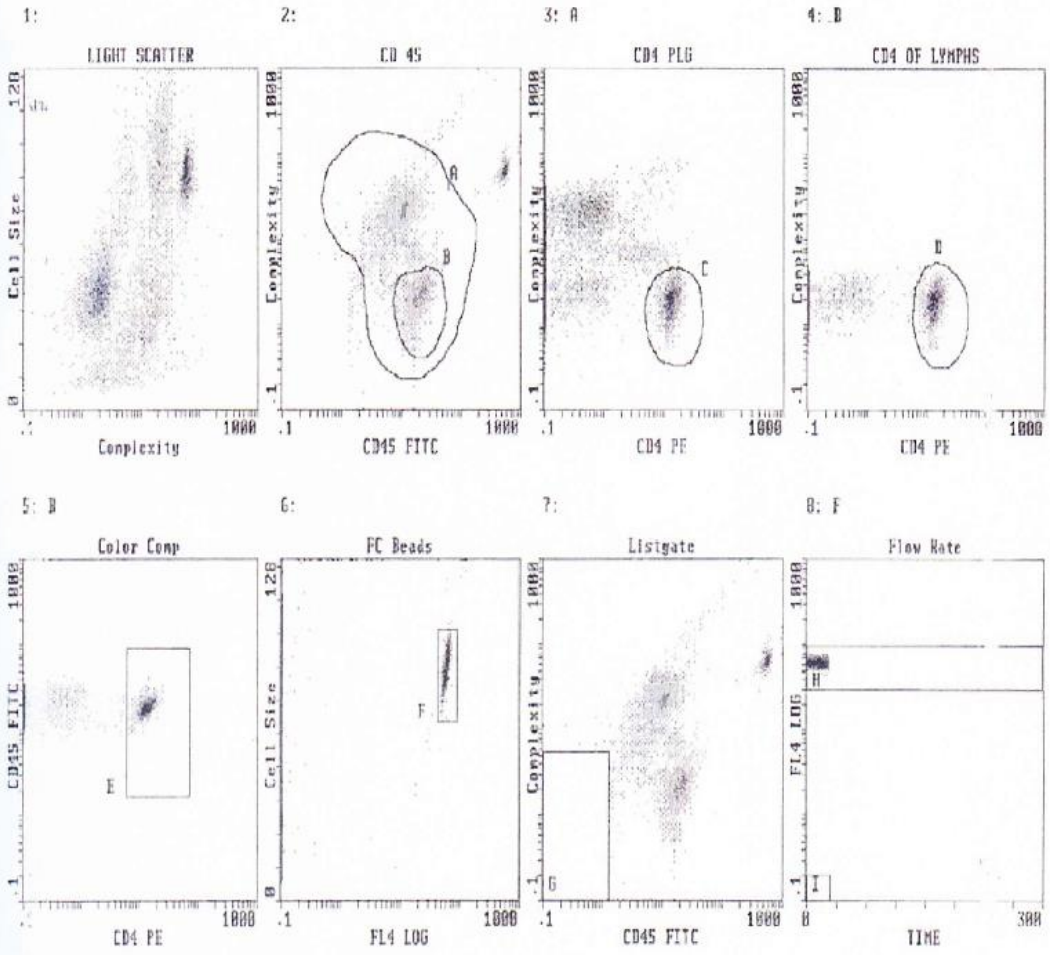
23Jan09 08:49:51
1-cdarv TEST
G0041941
4536036

OP ID: XL4MM

29 seconds, 15063 events

Initial cytosett. from prot. 1-cdarv TEST

Stop Count: 5000 events, histogram 4



Stats: Not Normalized, Listgating: Enabled, counts adjusted by: 958/2465

His	Region	ID	%	Count	Mn X	Md X	Mn Y	Md Y	FPCV%
2	A	TOTAL WCC	80.9	4738	12.0	13.1	6.44	6.65	126.83
	B	LYMPHS	33.2	1943	18.8	18.9	1.80	1.98	41.82
3	C	%CD4 OF WCC	18.4	870	13.2	13.2	1.72	1.78	42.54
4	D	%CD4 OF LYMPHS	44.3	860	13.3	13.2	1.72	1.78	41.86
5	E	COLOR COMP	44.4	863	13.3	13.2	17.5	17.5	21.75
6	F	CAL958	16.4	958	59.2	59.1	87.2	87.5	6.83
7	G	LISTGATE-	0.00	0	****	****	****	****	**
8	H	FLOW RATE	100	958	5.6	5.6	59.2	59.1	9.0%
	I	FCLOT7548027	0.00	0	****	****	****	****	**

Appendix C: Gant chart

Task	Duration	MONTH 1	MONTH 2	MONTH 3	MONTH 4	MONTH 5	MONTH 6	MONTH 7	MONTH 8	MONTH 9	MONTH 10	MONTH 11	MONTH 12
Literature Review	2 months												
Protocol preparation	3 months												
Ethics application	1 month												
Postgraduate Committee	1 month												
Funding Application	1month												
Specimen Collection	3 months												
Laboratory Activities	3 months												
Data Collection	3 months												
Data Analysis	2 months												
Writing Up	2 months												

Appendix D: Uncertainty of measurement

Table one summarizes the expected imprecision and bias for CD4 percentage and absolute counts.

Table 1: Expected imprecision and bias for CD4 percentage and absolute counts

Test	Test value	Bias	Imprecision
CD4 absolute	<100	~15 cells	<7%
	>100<200	~20 cells	<7%
	>200<400	~40 cells	<6%
	>400<700	~65 cells	<5%
	>700	~100 cells	<5%
CD4 percentage	N/A	<2%	<2%

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Appendix B: Post graduate committee approval letter

Dear Dr Pretorius, Dear Prof Mayne

The comments from the assessors on the revised protocol of Dr Pretorius area as follows:

- Much improved on original version
- Still hasn't clarified the sample size issues (i.e. 50 patients and 20 controls) or does the proposed post-hoc analysis (10 vs 10) address this? What if a larger proportion of controls get excluded so that "data matching" can't be done properly? Is it not easy to get HIV-negative patients on dialysis treatment?

- The aim of the study is confusing and not clearly stated. Maybe it should read as " to assess the trend of the CD4+ T-cell count in HIV infected patients with end stage renal disease receiving dialysis."

- Also who will be collecting the samples at the different hospitals (pre / post dialysis), because the candidate says that samples collected in wrong tubes, haemolysed samples and samples received >48hrs time from collection, will be rejected. It appears that nurses in the renal units performing dialysis might be collecting the samples, explaining the study to the patients and obtaining consent, something that the candidate should be doing!! Bearing in mind it is both HIV positive and HIV negative patients (matched controls).
- For HIVAN would the eGFR be increased as stated or decreased?

The protocol can be revised to the satisfaction of the supervisors and HOD.

Thando Mbolekwa
Postgraduate Research Administration
Faculty of Health Sciences
Second Floor of PVTobias Building
Wits University
Parkton, JHB

Office Tel: 0117172125
Email: Thando.Mbolekwa@wits.ac.za

Appendix C: Ethics clearance certificates

R14/49 Dr Melanie Pretorius

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170858

NAME: (Principal Investigator)	Dr Melanie Pretorius
DEPARTMENT:	Haematology and Molecular Medicine Chris Hani Baragwanath Academic Hospital Helen Joseph Hospital
PROJECT TITLE:	Factors influencing CD4+ T-cell Counts in HIV-infected Patients with End-stage Renal Disease
DATE CONSIDERED:	25/08/2017
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr E. Mayne, Dr E.L. Benade and Dr D. Lawrie
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	02/10/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review August and will therefore be due in the month of August each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 2/10/2017

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

6 February 2017

Dr M Pretorius
School of Pathology
Department of Molecular Medicine and Haematology
NHLS
Sent by e-mail to: melanie.pieters@gmail.com

Dear Dr Pretorius

Re: **Protocol Ref No:** M170858
Protocol Title: *Factors influencing CD4+ T-cell counts in HIV-infected patients with end-stage renal disease*
Principal Investigator: Dr M Pretorius

Thank you for your letter of 15 November 2017. I apologise for the slow turnaround.

I confirm that the addition of two new sites at CMJAH and Donald Gordon Medical Centre to Protocol No. M170858 has been noted and approved.

Thank you for keeping us informed and updated.

Yours Sincerely

.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

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Research Office Secretariat:

Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za.
Website:http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/