Statistical Analysis Plan

RHICCA study

Reactivation of **h**erpesviruses and **i**nflammation as **c**ardiovascular and **c**erebrovascular risk factors in **a**ntiretroviral initiators, in an African HIV population

The identification of modifiable viral and inflammatory risk factors for cerebrovascular and cardiovascular disease (CBD/CVD) in HIV-infected Malawian adults.

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1 Preface

This Statistical Analysis Plan (SAP) for "Reactivation of herpesviruses and inflammation as cardiovascular and cerebrovascular risk factors in antiretroviral initiators, in an African HIV population" (RHICCA) describes and expands upon the statistical information presented in the protocol.

This document describes all planned analyses and provides reasons and justifications for these analyses. This SAP will follow internationally accepted guidelines published by the American Statistical Association[1] and the Royal Statistical Society[2] for statistical practice.

This document contains a review of the study design, general statistical considerations, comprehensive statistical analysis methods. Any deviation from this SAP will be described and justified in the final study report. The reader of this SAP is encouraged to also review the study protocol for details on conduct of the study.

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3 List of abbreviations

AAC	All-available cases
ACR	Albumin-creatinine ratio
AIC	Akaike information criterion
ART	Antiretroviral therapy
BIC	Bayesian information criterion
CHCs	Blantyre City Community Health Centres
CoM	Malawi College of Medicine (University of Malawi)
PWV	(carotid femoral) Pulse wave velocity
CBD	Cerebrovascular Disease
CHC	Community Health Centre
cIMT	Carotid intimal medial thickness
CMV	Cytomegalovirus
COMREC	College of Medicine Research Ethics Committee
Co-PI	Co-Principal Investigator
Co-I	Co- Investigator
CRF	Case record form
CVD	Cardiovascular disease
EBLUP	Empirical best linear unbiased predictor
eGFR	Estimated glomerular filtration rate
GCP	Good clinical practice
GEE	Generalised estimating equations
GLM	Generalised linear model
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IQR	Inter-quartile range
IRB	Institutional Review Board
IRIS	Immune reconstitution syndrome
LMM	Linear mixed model
lowess	Locally weighted scatterplot smoothing

LTFU	Lost to follow-up
MAR	Missing at random
MCAR	Missing completely at random
MI	Myocardial Infarction
MLW	Malawi-Liverpool-Wellcome Clinical Research Programme
PCR	Polymerase Chain Reaction
ODK	Open Data Kit
OR	Odds ratio
PI	Principal Investigator
PVD	Peripheral vascular disease
QECH	Queen Elizabeth Central Hospital
QQ plot	Quantile-quantile plot
REALITY	Reducing Early Mortality on antiretroviral therapy (Clinical Trial)
SAP	Statistical Analysis Plan
SHIELD	Study into HIV, Immune activation and endothelial Dysfunction (Clinical Study)
SMS	Short message service
SOPs	Standard Operating Procedures
SSA	Sub Saharan Africa
RR	Risk ratio / relative risk
TDF/3TC/EFV	Tenofovir/Lamivudine/Efavirenz
VZV	Varicella Zoster
WHO	World Health Organisation

4 Introduction

In Africa, rising rates of cerebrovascular and cardiovascular disease (CBD/CVD) are intersecting with an ageing HIV-infected population. In Western countries, HIV is associated with a 50% increased risk of CVD compared to HIV-uninfected populations, due to both anti-retrovirals (ART) and HIV per se. High quality prospective studies are now needed to determine if HIV-infected patients in Africa are at increased risk of CBD/CVD and to identify factors associated with this risk. The RHICCA study will test the hypothesis that immune activation, and in turn dysfunction, driven by HIV and reactivation of latent herpesvirus infections leads to increased CBD/CVD risk in Malawian adults aged $\geq$ 35 years.

We will conduct a single-centre 36-month prospective cohort study in 800 HIV-infected patients initiating ART and 190 HIV-uninfected controls in Blantyre, Malawi. Patients and controls will be recruited from government ART clinics and the community, respectively and will be frequency matched by 5-year age band and sex. At baseline and follow-up visits, we will measure carotid intima thickness (cIMT) and pulse wave velocity (PWV) as surrogate markers of arterial damage, and thus CBD/CVD risk. Our primary exposures of interest will be prospectively measured; these include herpesvirus reactivation, HIV viral load, and markers of systemic inflammation and endothelial function. Multivariable regression models will be developed to assess the study's primary hypothesis. Occurrence of clinical CBD/CVD will be assessed as secondary study endpoints.

The study will be carried out in accordance with guidelines on protection of human subjects in the 2008 Declaration of Helsinki; it was approved by the University of Malawi College of Medicine and the Liverpool School of Tropical Medicine ethics committees.

5 Study objectives, outcomes, exposures

Objectives

This study will test the hypothesis that immune activation, and in turn dysfunction, driven by HIV and reactivation of latent herpesvirus infections leads to increased CBD/CVD risk in adults aged ≥ 35 years in SSA. We will address this through the following objectives;

1) To determine if vasculopathy progression or occurrence of new-onset clinically significant vasculopathy is higher in adults aged ≥ 35 years with HIV infection on ART compared to those without HIV
2) To determine if vasculopathy progression or occurrence of new-onset clinically significant vasculopathy is higher in adults aged ≥ 35 years with viral antigenaemia or chronic immune activation compared to those without viral antigenaemia or chronic immune activation. Specifically, we will determine if vasculopathy is higher:
    a. in ART patients with reactivated latent herpes viral infection, compared to those without reactivated latent herpes viral infection.
    b. in ART patients in the top 20% of markers for immune activation, immune senescence, inflammation or coagulation compared to the bottom 20%
    c. in ART patients with incomplete virological suppression or virological resurgence of HIV, compared to those with suppressed HIV plasma viral load

The secondary study ssobjectives are to determine if viral antigenaemia or chronic immune activation increase occurrence of the following clinical events: 1) stroke, 2) myocardial infarction (MI), 3) angina (excluding MI), 4) peripheral vascular disease (PVD), 5) all-cause death/vascular-related death and 6) immune reconstitution vasculopathy.

Primary outcomes

Primary outcomes are the progression and occurrence of clinically significant levels of surrogate markers of CBD/CVD risk, namely carotid femoral pulse wave velocity (PWV) and carotid intima media thickness (cIMT).

PWV will be measured at each 6-monthly study visit and cIMT will be measured at baseline and 24 months.

PWV measurement will be in accordance with expert consensus guidelines[3], using standardized study protocol on the Vicorder system. PWV > 10 m/s will be set as the threshold of clinically significant CVD risk[3]. Far wall measurements of the common carotid artery at least 10 mm below the carotid bifurcation[4] will be measured at baseline and at 24 months with a high-resolution B-mode system operating in the black and white mode with a linear ultrasound transducer at frequencies of greater than 7 MHz. A value of > 0.90 mm will be set

as clinically significant cIMT[3]. Plaques will be defined by focal wall thickening > 50% than the surrounding vessel wall or a focal region with an IMT measurement ≥1.5 mm that protrudes into the lumen[4]. Operators will be trained by taking repeated measurements, but only the final measurements will be recorded and used in the analysis.

Secondary outcomes

Secondary outcomes are the following clinical events: 1) stroke, 2) myocardial infarction (MI), 3) unstable angina, 4) peripheral vascular disease (PVD), 5) all-cause death/ vascular death and 6) Immune Reconstitution Syndrome (IRIS) vasculopathy (which we define as a new clinical event within 6 months of ART initiation, including surrogate events measured by PWV and clinical vascular events defined as MI / stroke and vascular related deaths). Changes in PWV or endothelial activation at 6 months post ART initiation will be interpreted as subclinical vascular IRIS events. These outcomes will be assessed through active surveillance in QECH inpatient wards for admissions of study participants. QECH offers care free at the point of delivery and is the only government inpatient facility in Blantyre. To improve capture of clinical outcomes, we will conduct brief telephone interviews with study participants about CBD/CVD symptoms and hospitalizations between study visits and facilitate unsolicited participant self-report. All suspected clinical events and deaths in study participants will be reviewed by an independent endpoint review committee (ERC) consensus will be achieved based on a structured diagnostic criteria template (see protocol). The format of reporting and diagnostic criteria will be based on modifications of the INSIGHT network clinical templates. When death occurs unexpectedly in the community, verbal autopsy will be performed to ascertain the cause[5].

Exposures

The exposure for primary pobjective 1 will be HIV status. Yearly HIV rapid tests in HIV-uninfected adults will be performed to exclude those with new HIV infections.

Potential confounding and mediating factors will be measured in study participants. This will include demographic and socio-economic factors, lifestyle and behavioural factors (e.g. cigarette smoking and alcohol consumption), chronic co-morbidities (captured at baseline), cardiometabolic, renal and haematological factors (i.e. cholesterol, HBA1C, full blood count, creatinine in urine and serum, body-mass-index, waist-to-hip ratio and history of hypertension, diabetes and hypercholesterolemia). Blood pressure will be measured at all study visits. Although vascular IRIS will be considered as a primary endpoint, non-vascular IRIS will be defined as a risk factor. Where feasible, we will conduct PCR tests for common causes of IRIS in blood or cerebrospinal fluid (CSF) samples. ART adherence and change in ART will be assessed at all study visits through an 'ART master card', which is routinely maintained on all ART patients in Malawi.

By default, all adjusted analyses will include the following covariates: age, sex, socio-economic factors, current smoker, current or former alcohol consumption, co-morbid respiratory disease, previous or current kidney disease, previous or current heart failure, previous or current high cholesterol, previous or current diabetes, previous or current hypertension, obesity (defined by BMI), abdominal obesity (defined by WHR), HIV status, CD4+ count, viral load, VZV and CMV antibodies.

For objectives 2b and 2c, markers of viral antigenaemia and immune inflammation will be measured at baseline, 6, 12, 24 and 36 months in ART patients, and at baseline and yearly thereafter in HIV-uninfected adults. For primary study objective 2a, reactivated latent herpes viral infections will be assessed by quantification of VZV and CMV IgG antibodies at baseline in HIV-uninfected controls and at baseline and 6 months in HIV-infected participants. We will establish the risk associated with prior reactivation (i.e. reactivation occurring in the 6 months before starting ART) from baseline measurements, and sustained reactivation (i.e. those that continue to have a high titre from measurement at baseline to 6 months after ART initiation). Hyperactivation of B cells may result in an expansion of polyclonal antibodies and thus an overestimation of virus-specific antibody titres. To address this issue and make appropriate adjustments to antibody titre estimates we will 1) measure more than one herpesviruses (i.e. CMV, VZV) and 2) measure total IgG to determine if an increase in specific viral IgG titre is independent of a total IgG response.

For primary objective 2b, seven markers of immune activation, inflammation, coagulation and endothelial activation will be measured. Quantitative cell surface immunophenotyping will be performed for CD4+ and CD8+ T-cell activation (HLA-DR) and senescence (CD57) will be conducted in all participants patient subset. In all study participants, at baseline, 6, 12, 24 and 36 months we will measure soluble markers associated with systemic inflammation (IL-6, hsCRP), activation of coagulatory pathways (D-Dimers) and endothelial activation (sICAM-1 and sVCAM-1).

For primary objective 2c incomplete viral response and viral rebound of HIV will be measured by quantitative PCR in ART patients. A binary variable for incomplete viral response will be set to 1 in patients with two consecutive viral load measurements exceeding 1000 copies/ml within a 6-month interval after at least 24 weeks on ART[6], and otherwise set to 0. A binary variable for viral rebound will be set to 1 if there is detectable viral load exceeding 1000 copies/ml at 12 months after having achieved complete viral suppression; and otherwise set to 0. HIV viral load will be measured in ART patients at 0, 6 and 12 months.

6 Investigational plan

Study design

To address objective 1, we will conduct a single-centre 36-month prospective cohort study in 800 HIV-infected patients initiating ART and 190 HIV-uninfected adults aged ≥ 35 years. HIV-infected and HIV-uninfected participants will be frequency matched by 5-year age band and sex. On a 6-monthly basis, we will measure markers of viral infection, inflammation and endothelial function along with surrogate markers for CBD/CVD risk.

Study Setting

This study will recruit ART patients from the ART Clinic of Queen Elizabeth Central Hospital (QECH), and ART clinics in several Blantyre City Community Health Centres (CHCs). These clinics represent the highest-volume government ART facilities in Blantyre; they offer free ART and are where most ART patients receive care. Collectively, they initiate over 100 HIV patients aged ≥ 35 years onto ART each month. HIV-uninfected adults will be selected by two-stage random sampling (of households and individuals within households) from a previously enumerated sampling frame in the CHC catchment areas[7]. All study procedures will be conducted at QECH, which is located adjacent to the Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW), an internationally-renowned research facility that will enable high quality laboratory analysis and GCP-compliant data management. QECH also hosts a 0.35T MRI imaging facility, which will contribute to characterising our secondary endpoints.

Study Participants

Study inclusion criteria are: a) aged ≥ 35 years and b) resident in Blantyre. HIV infected patients must further be: c) ART-naïve or initiated ART <10 days prior to enrolment and d) initiating standard first line ART (in Malawi this is: TDF/3TC/EFV). Adult controls must further be: e) HIV uninfected. Study exclusion criteria are: f) clinical history of CBD/CVD, g) pregnant, h) critically ill or have symptomatic anaemia at enrolment and i) enrolled in an intervention study.

Justification of study inclusion and exclusion criteria is as follows; in many populations CVD/CBD risk rises sharply with age starting at about age 35[8], thus individuals aged 35 and older will be eligible (recruitment of participants aged 35 -39 will be limited to 15% of the study sample to avoid overrepresentation). Restricting recruitment by age will enable this study to have greater statistical power. For clarity of aetiologic inference, the study will assess risk of new onset clinically significant vasculopathy not associated with pregnancy and thus exclude patients who are pregnant or with a history of CBD/CVD. To eliminate confounding

by ART regimen, patients must initiate on standard first line ART (> 90% of ART patients in Blantyre do this). Critically ill patients are excluded primarily for ethical reasons.

Data Collection

Two-stage screening will be conducted to find and recruit potential study participants. A trained field worker will first screen to assess eligibility for criteria (a) - (c) in patients attending pre-ART counselling sessions, and in individuals from randomly selected households in the community. Eligible participants will then be referred to the study office at QECH to complete screening for criteria (d) - (i) and if eligible, consented to participate in the study. At study visits, a tablet-based, standardized Open Data Kit (ODK) case report form (CRF) will be administered in one-on-one interviews by a study nurse to capture demographic and clinical data. Study visits also include measurements of PWV and cIMT. To measure cardiometabolic factors, inflammation, markers of HIV disease progression (CD4+ count, CD4+ / CD8+ ratio, viral load), immune activation and latent infection, we will collect up to 30 mls of whole blood; this quantity of blood is safe for adults, according to WHO guidelines on best practices for phlebotomy[9]. An albumin-creatine ratio (ACR) dipstick test will be used to test for creatinine, proteinuria and glucose and thus evaluate renal function. The estimated glomerular filtration rate (eGFR) will be calculated from the creatinine measurement. In a participant subset, an electrocardiogram and echocardiogram will be performed at baseline, 6 months and 2 years, as well as in any participant experiencing a clinical event suggestive of a cardiac aetiology. This approach will capture symptomatic, silent MIs and heart dysfunction. Abnormal test results will be referred for clinical follow-up in accordance with standard clinical practice at QECH. Participant visit samples will be transported from the study office to the MLW laboratory, which is located adjacent to the QECH hospital campus, following standard operating procedures. To facilitate participant retention and clinical referrals, study participants will be contacted every 3 months to assess occurrence of clinical events. Daily upload of electronic data, including date of next scheduled visit will be used to produce real time daily reports of scheduled participants. Participants who miss a scheduled study visit will be contacted by phone and/or visited at home to assess their willingness for study participation; SMS messaging will be used for appointment reminders.

Sample Size

The required sample size for the study's primary objectives is 800 HIV-infected patients and 190 HIV-uninfected adults using standard, normal distribution approximation sample size formulas for comparing proportions in two groups of unequal size and based on the following assumptions: **a)** 18.4% of HIV positive study participants have abnormal PWV at baseline. In our ongoing studies of vasculopathy in HIV-infected patients, 18.4% aged $\geq$35 years have a PWV (>12 m/s), **b)** 20% of both HIV-infected patients and HIV-uninfected adults will be lost to

follow-up, including by death and HIV sero-conversion[40 41]. **c)** The minimum relative risk (RR) of interest is 2 for objective 1 and 1.8 for objective 2. **d)** Cumulative risk of clinically significant vasculopathy over study follow-up is 18.4%. This is based on study data cited in (a). **e)** For objectives 2a)-c)**,** the exposure prevalence for each risk factor is 20%. **f)** Statistical tests will have 80% power based on a 2-sided test with; α=0.05. Testing of hypotheses80 deaths occurring during the [10,11].

7 General considerations

The reporting of this study will be prepared in accordance to the STROBE[12] guidelines

All continuous data variables will be summarized using the following descriptive statistics: N (size of relevant analysis population), n (size of analysis population without missing values), arithmetic / geometric mean, standard deviation (SD), median, 25th percentile value (P25), 75th percentile value (P75) and interquartile range (IQR), minimum and maximum. The geometric mean will be reported for log-transformed variables. The proportion of observed levels will be reported for all binary and categorical measures. When appropriate, corresponding exact 95% confidence intervals (CIs) for proportions will be included.

For all longitudinally measured, continuous primary and secondary outcomes, time plots of the individual response profiles will be produced and inspected. Further, to expose trends in the data, we will add locally weighted scatterplot smoothing (lowess) lines to the time plots. This is for descriptive and exploratory purposes only and we will not estimate confidence intervals for the lowess curves as these will be biased to be too narrow given the longitudinal nature of the data. Kaplan-Meier (KM) curves for all-cause and vascular death will be produced and inspected, particularly for differences between groups defined by sex and exposures for objectives 1 and 2a-c (HIV status, reactivated latent herpes viral infection, top & bottom quintiles of immune activation markers, incomplete viral suppression or resurgence).

7.1 Timing of analyses

There will be 3 stages of analysis: i. at baseline, once all recruited participants have had baseline data collected, ii. at 6 months, once all recruited participants have had their 6 months follow-up visit (resp. determined to be lost to follow-up for this visit), iii. at study conclusion at 36 months, once all recruited participants have finished their last scheduled follow-up visit (resp. have been determined as lost to follow-up for this visit) and no further data is collected as part of the study.

7.2 Missing data

All efforts will be made to collect complete data on all study participants. However, as for all longitudinal studies, it is expected that some participants will be lost to follow-up or miss some of the follow-up visits for a variety of reasons. In fact, by protocol it is expected that up to 20% of HIV-infected and up to 10% of HIV-uninfected participants will be lost to follow-up and the sample size calculation has taken this into account. All primary analyses will be performed using multiple imputation (MI; valid under missing-at-random (MAR), and can also be applied under less strict assumptions). For sensitivity analyses, we will use all-available-

cases (AAC) which assumes missing-completely-at-random (MCAR) , direct likelihood and fully Bayesian models and, for generalised estimating equations (GEE), weighted GEE (all assuming MAR). If the number of missingness patterns is sufficiently small, we will also use pattern mixture models which can be used under the general missing-not-at-random (MNAR) setting, but make additional identification assumptions.

7.3 Statistical significance and multiple testing

All statistical hypothesis tests will use a two-sided significance level of $\alpha = 0.05$, unless stated otherwise. As we will fit several models for several outcome variables, we will report both unadjusted p-values and p-values adjusted for multiple testing according to the Benjamini-Hochberg[13,14] procedure. The multiplicity adjustment is done at final analysis for all statistical tests involving the primary outcomes PWV and cIMT.

7.4 Covariates, subgroups, model selection

Covariates to be included in all primary analysis models and all subgroups to be analysed as part of the primary analyses will be pre-specified in this SAP. Any changes to covariate sets or subgroups will be detailed in the analysis report together with the reason for doing so, as well as clarity on all models that were fitted to the data and all subgroup analyses that were done. Reasons for including covariates are based on clinical expertise and published literature. This is to avoid the undesirable statistical properties of p-value based variable selection methods such as stepwise selection methods[15].

For secondary and exploratory analyses, wherever variables need to be selected for a model, we will prefer the use of variable reduction techniques that do not involve the response variable to avoid overfitting.

Transformation of response or predictor variables, such as log or quantile transformations, will be applied if required to satisfy model assumptions. Should the linear models described above indicate clear non-linearities or abrupt changes in slopes, we will extend these models through the use of restricted cubic splines[16] and, potentially, generalised additive models[17].

For mixed models, we will use likelihood ratio tests, with the appropriate degrees of freedom, to determine whether or not to include random effects beyond random intercepts.

For primary analyses we will only consider unstructured covariance matrices for the random effects in mixed models and diagonal covariance matrices (i.e. homoscedasticity) for the residuals. For exploratory analyses we may investigate other correlation structures in the mixed models.

Generalised estimating equations (GEE) models will be fitted using exchangeable correlation matrices.

7.5 Assumption verification and model diagnostics

Verification of the normality assumption for t-tests will be done by visual inspection of histograms and quantile-quantile (QQ) plots. Formal tests for normality will be avoided as in large samples these will reject the null hypothesis of normality even for small deviations from normality (and in large samples with only small deviations from normality, the Central Limit Theorem will guarantee that t-test results are valid).

We will use standard model diagnostic techniques for the generalised linear models we will develop. Specifically, we will check linearity, additivity and distributional assumptions for all generalised linear models (GLMs) and also check for influential or outlying observations. For the first three of these checks, we will construct residuals plots (against the predictor variables and fitted values for linearity and additivity, against fitted values and histograms for distributional assumptions if applicable). For predictors for which we suspect non-linear effects we will use restricted cubic spline functions[16] to shed light on whether these variables should be modelled differently and, if yes, how those variables should be modelled. To check for influential observations, we will check first for outliers by looking at residuals against observed values, then by computing deletion diagnostics: we will first compute an aggregate measure, Cook's D distance[18], and then investigate the sensitivity of individual regression coefficients to leaving out individual observations (DFBETAs[19]).

For the linear mixed models (LMMs), we will also perform residual diagnostic checks, again checking linearity, additivity and distributional assumptions. We will also compute influence diagnostics, check for both outlying subjects and outlying observations and perform diagnostic checks on the random effects. Given the nature of LMMs, this means we will compute several types of residuals: marginal residuals, studentised conditional residuals and empirical best linear unbiased predictors (EBLUPs). Linearity will be assessed by checking plots of marginal and conditional residuals against explanatory variables. Distributional assumptions will be verified by checking histograms of the conditional residuals and plots of these against fitted values. Outlying subjects will be checked for by plotting EBLUPs against subjects and outlying observations by looking at conditional residuals against observations. For influential observations we will compute both likelihood and restricted likelihood distances as well as Cook's D for both the vectors of fixed and random effects.

For GEE fitted models, we will use similar diagnostic checks as for the GLMs, but use studentized Pearson residuals and extensions to GEE of the standard diagnostic tools described above.

7.6 Reporting conventions

P-values ≥0.001 and ≤0.999 will be reported to 3 decimal places; p-values less than 0.001 will be reported as "<0.001". The mean, standard deviation, median, IQR and other statistics will

be reported to one decimal place greater than the original data. Minimum and maximum values will use the same number of decimal places as the original data. Proportions will be presented as two decimal places; values greater than zero but <0.01 will be presented as "<0.01". Percentages will be reported to 2 decimal places; values greater than zero but < 1% will be presented as "<1%"; values greater than 99% but less than 100% will be reported as >99%. Estimated parameters, not on the same scale as raw observations (e.g. regression coefficients) will be reported to 3 significant figures.

7.7 Technical details

All analyses and figures will be performed using the R environment for statistical computing and graphics[20] and associated packages. Bayesian models will be implemented using JAGS[21] or Stan[22] via an R interface. All relevant R, JAGS and Stan code will be made available, and version numbers of R, JAGS, Stan and associated packages that were used will be reported along with the analysis results for full reproducibility.

8 Analysis

While we will develop models that adjust for potentially confounding or mediating variables, we will also perform unadjusted analyses. Except for baseline summary analyses, all analyses comparing HIV-infected and -uninfected participants will be adjusted for age and sex due to the matched study design.

8.1 At baseline

We will compute summary statistics for all outcome and exposure variables at baseline as described in Section 7. Further baseline values of confounding or mediating factors (as defined in Section 5), exposure and outcome variables will be compared between the HIV-infected and -uninfected adults. For outcome variables these comparative analyses will be adjusted for 5-year age bands and sex. All other analyses will be unadjusted. We will perform similar comparative analyses between ART patients with and without reactivated latent herpes viral infection and between ART patients in the top and bottom quintiles of immunological markers. Virological suppression groups of ART patients as required for objective 2c cannot yet be determined at baseline analysis and hence these groups will not be compared at baseline.

Unadjusted analyses will consist of paired t-tests or Wilcoxon signed rank tests (depending whether the data are normally distributed or not) for continuously measured variables and Chi-Squared or Fisher's exact tests (depending on contingency table cell counts) for binary and categorical variables.

Adjusted analyses will be conducted using GLMs.

We will also perform a range of exploratory analyses at baseline: specifically, we will interrogate associations between immunological markers (CD4+, IL-6, ICAM, CD163), BMI, blood glucose, eGFR and the primary outcome variables (PWV, cIMT). These analyses will be stratified by HIV status, but we will also do a combined analysis, adjusting for HIV status.

Additional analyses to the above may be performed, but again these will be strictly exploratory and corresponding results labelled as such.

8.2 At 6 months

The analysis using data at 6 months for participants serves 2 purposes:

1. Characterise new onset vasculopathy in HIV-infected participants that have initiated ART treatment at baseline (vascular IRIS).
2. Define vasculopathy outcomes for the final analysis.

We will summarise vascular IRIS events in the ART population and stratify these summaries by potential risk factors (sex, age bands, reactivated latent herpes virus infection, incomplete virological suppression or virological resurgence, …).

We will also test for differences in vascular IRIS between groups defined for objectives 2a-c using GLMs (either log-binomial or logistic regression models, depending on convergence of the log-binomial models), regressing new onset vasculopathy at 6 months against binary indicators for two binary variables for herpesvirus reactivation at 0 and 6 months (objective 2a), against a binary indicator for top or bottom quintile of immunological markers (objective 2b) and against a binary indicator for incomplete virological suppression (objective 2c) respectively.

Summarising the vasculopathy events at 6 months will allow us to determine which events are likely to be observed in large enough numbers for a meaningful analysis at study conclusion.

8.3 At study conclusion (36 months)

8.3.1 Primary analyses

The study will run for 36 months from recruitment start to guarantee all participants to be followed-up for at least 24 months, but some of the earlier study recruits can have follow-up data up to 36 months. For the change from baseline analyses using only two time-points we will use the outcome at 24 months, not 36 months.

For objective 1 we will develop three regression models. Two GLMs will be developed to compare mean progression of arterial damage from baseline in HIV-infected ART patients and HIV-uninfected adults. These models will regress change from baseline in PWV, respectively cIMT, on HIV status. A log link function may be used if required to satisfy model assumptions, otherwise an identity link function will be used. The GLMs will include baseline PWV (respectively cIMT) as a covariate. We will develop a third model to estimate the RR and population attributable fraction of new-onset arterial damage in HIV-infected patients compared to HIV-uninfected adults. This will be either a log-binomial or a logistic[i] model (depending on convergence of the log-binomial model) which regresses a binary factor for new-onset arterial damage on HIV status in all participants without clinically significant arterial damage at baseline. This model will include the same covariates as the two GLMs for PWV and cIMT.

For objective 2a, a set of GLMs will be developed to compare mean progression of vasculopathy in HIV-infected ART patients with and without reactivated latent herpes viral

[i] Note that a logistic regression model will estimate odds ratios (ORs) not RRs. However, exposure (HIV status) has been fixed, not outcome (new onset vasculopathy), hence RRs can be derived from the ORs and the risk of new-onset arterial damage in the HIV-uninfected group.

infection. These models will regress change from baseline in PWV, respectively cIMT, on two log-transformed variables for antibody titres of CMV and VZV, respectively.

For objective 2b, we will again fit a set of GLMs, with change from baseline in PWV as response variable, this time to investigate if, in HIV-infected ART patients, there is an association between progression of vasculopathy and immune activation and inflammation biomarkers (IL-6, ICAM, CD163). Specifically, for each marker, we will regress PWV on marker quantiles. We will also compute correlations between markers to identify independent markers. After having built models for each marker, we will then develop comprehensive multiple regression models for PWV and cIMT with multiple independent markers as predictor variables.

For objective 2c, we will proceed as for objective 2a, but comparing HIV-infected ART patients with incomplete virological suppression or virological resurgence of HIV to those with suppressed HIV plasma viral load.

In addition to these analyses, given the repeated measurements for PWV, immune activation, inflammation markers, we will extent the GLMs for PWV to LMMs taking full account of the longitudinal nature of the data. Mixed models will also handle deviations from the visit schedule in a principled fashion and use all available data for drop-outs and remain valid under MAR. By using both mean-centered predictor variables and mean predictor values for time-varying variables in the models, we can tease apart cross-sectional and longitudinal effects, should they differ. All LMMs will include time since baseline as a covariate, as well as an interaction term for exposure of interest and time since baseline. In the case a log link function is required for PWV in the GLMs, we will fit marginalised models using GEE instead of LMMs.

In the case of LMMs, we will start with random subject-level intercepts only, then investigate whether adding random slopes for the main predictor variable(s) improves model fit.

8.3.2 Secondary / exploratory analyses

For secondary study objectives, we will use univariate methods to assess the frequency of clinical events within exposure strata. If there are sufficient numbers of clinical events we will develop Poisson or negative binomial regression models (depending on model fit) for each clinical event type to compare participant groups defined in primary objectives 1 and 2a-c. We will also analyse a compound outcome for the number of occurrences of any of the secondary clinical events. The exact definition of this compound variable will be determined at the 6-month analysis.

As some of the exposures are time dependent, we will also use log-binomial or logistic GEE models to investigate the association between exposure variables and clinical events (individual types of events as well as the compound outcome of any event). As previously we will only do these analyses if there are sufficient numbers of recorded clinical events. We will

also determine the time of greatest vasculopathy risk in ART patients. Since new onset vasculopathy is determined by PWV and cIMT (measured only at defined visits), we will compute this risk for each of the discrete time points where PWV or cIMT are recorded.

We will also use time-to-event models, specifically Cox proportional hazard models, to investigate associations between all-cause mortality and exposures.

Finally, to identify risk groups that are potentially not well characterised by the measured exposure variables and to confirm any associations we have found, we will perform unsupervised group-based multi-trajectory modelling of multivariate longitudinal patient profiles[23].

Also, while the study may be too limited to develop and validate a risk score for new onset vasculopathy in HIV positive individuals in Malawi, we will explore the predictive performance of existing risk scores, such as the Framingham Risk Score[24,25], within our cohort and characterise the major differences of such scores with an exploratory risk score developed from our data.

9 References

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