Statement of Hypotheses and Specific Aims:

Hypertension is a leading risk factor for stroke, heart diseases, and renal failure imposing an enormous health care burden.[1] **Despite the proportion of hypertensive HIV positive adults (PWH) exceeding 35%** in recent global estimates, the etiology of hypertension in HIV remains unclear[2]. It is becoming increasingly evident based on studies on the general population that hypertension is an inflammatory process that involves transmigration and accumulation of both innate and adaptive immune cells into the vasculature where they release cytokines and produce oxidative stress to promote vascular damage.[3] Our preliminary data demonstrated that PWH on stable ART have elevated levels of markers of microbial translocation, vascular inflammation and immune activation independent of other comorbidities and HIV-specific factors[4, 5]. As such, we **hypothesize** that the development of HIV-related hypertension may be driven in part by the persistent low grade chronic immune activation leading to vascular tissue damage, and that microbial translocation may contribute to this dysregulated immune response.

Sub-Saharan Africa (SSA) region has the highest burden of both HIV and HIV-associated CVD mortality, yet very few studies have investigated the pathogenesis of HIV-related hypertension.[6] Such studies are critically needed in SSA because studies in the US and Europe identified important differences in the pathogenesis and treatment responses of hypertension among different ethnic groups that could only partly be explained by lifestyle or dietary influences suggesting that the mechanisms may vary by ethnicity.[7] Moreover, Africans, regardless of their HIV status are exposed to distinct risk factors such as indoor air pollution, poor sanitation, and infections that can potentially modulate the immune system and drive the development of hypertension.[8-10] Identifying important risk factors for hypertension among Africans, particularly differences between those with and without HIV infection, will be essential in the identification of novel and important anti-hypertensive targets that would aid in the development of new therapeutic treatments for hypertension.

The **primary objective** of this study is to define immunologic and inflammatory contributors of hypertension in PWH on stable ART in western Kenya. This project will leverage resources of a previous NIH-funded study (R21TW010459: Farquhar) and an ongoing K01 funded study (K01HL147723: Temu) reporting on CVD risk factors among PWH and HIV-negative adult men and women (HIV-) in western Kenya, including participant recruitment, clinical data and bio-specimen collection. We will conduct a nested study and use plasma and PBMC from 245 PWH and 166 HIV- to measure a range of immunological biomarkers selected for their association with CVD in uninfected populations.

Hypothesis: *PWH on stable ART will have significantly greater markers of innate and cellular immune activation compared to their age/sex matched HIV- and microbial translocation and co-infections such as latent TB may contribute to the process.*

Aim 1a: Measure and compare biomarkers of innate immune activation including interleukin [IL]-6 among PWH and HIV- in western Kenya and explore drivers for differences. Predictors to be evaluated include latent tuberculosis, traditional CVD risk factors, microbial translocation markers (*lipoprotein binding protein and* β-*glucan),* and HIV-related characteristics such as $CD4^+$ T cell count and ART.

Aim 1b: Measure and compare markers related to cell-specific and global T cell immune activation, including $CD8^+$T cell expression of activation markers (CD38, HLA-DR), as well as plasma cytokines indicative of Th1 , Th2, and Th17 immune response.

Hypothesis: *Persistent innate and cellular immune activation promotes hypertension in PWH, independent of traditional risk factors and HIV specific risk factors.*

Aim 2a: Determine if IL-6 and other markers of innate immune activation are positively associated with hypertension in virally suppressed PWH on stable ART.

Aim 2b: Determine if the percentage of CD38+ and HLA-DR+ CD8+ T cells and other markers of and other markers of adaptive immune activation are positively associated with hypertension in PWH on stable ART.

IMPACT: This innovative and important exploratory study on a well characterized cohort will reveal mechanisms leading to hypertension in PWH on stable ART and non-infected Africans. In addition, it will provide pilot data for Dr. Temu for the design of future studies to better understand and prevent hypertension in SSA, including development of risk scores that incorporate immunological markers and intervention trials to improve HIV treatment outcomes by minimizing non-AIDS related comorbidities.

RESEARCH PLAN
BACKGROUND AND SIGNIFICANCE

Studies from high-income countries suggests that HIV infection may mediate systemic immune activation and inflammation which contribute to the increase risk for cardiovascular and metabolic disease including hypertension (HTN)[11]. This proposal will rigorously test if these parameters differ between hypertensive PWH versus non-hypertensive PWH on ART, as well as between hypertensive PWH and non-infected subjects. These results will inform guidelines for prevention and treatment for hypertension in PWH, which may differ from uninfected subjects, and enable design of interventions to mitigate hypertension risks in treated HIV in Africa.

A1. PWH are at increased risk of cardiometabolic diseases commonly associated with systemic immune activation inflammation. PWH on ART have an approximately 2-fold higher risk of incident CVD compared to their age-sex matched uninfected counterparts with much of the burden seen in SSA[11-14]. Hypertension is a leading risk factors for cardiovascular diseases in HIV. It is estimated that 23% of PWH in Kenya are hypertensive[15]. The pathophysiology of essential HTN remains incompletely understood and is currently attributed to the interplay of genetic and conventional cardiovascular risk factors. However, recent studies have shown that only 4% of the variance in systolic blood pressure can be explained by genetic risk variants and while lifestyle factors such as obesity are known to be important for the pathogenesis of HTN, a UK study revealed that it can only modify blood pressure by up to 5mm Hg depending on genetic risk. These findings imply that other factors may contribute to the process. There is now more evidence to support the role of activated immune cells, and their products (cytokines and reactive oxygen species) in hypertension resulting in endothelial injury among other processes that cause vascular damage.[3] A study by White et al demonstrated that athymic nude mice did not maintain HTN after renal infarction. Similarly, studies in human have also demonstrated the role of the innate and adaptive immune systems in the development of HTN in the general population[16, 17] However, it is not known which innate and adaptive immune factors contribute HTN in PWH.

A2. HIV may contribute to chronic immune activation and HTN through microbial translocation. Heightened systemic inflammatory processes contribute to the development of cardiovascular diseases (CVD) in PWH[18]. The etiology of chronic immune activation observed among PWH is not fully understood and likely multifactorial. Gut blood barrier disruption following mucosal $CD4^+$ T-cell depletion in PWH results in chronic entry of endotoxins including lipopolysaccharide (LPS) into the systemic circulation may contribute to persistent systemic immune activation[19]. LPS interacts in the bloodstream or in tissues with CD14-Toll-like receptor 4 complex on monocytes/macrophages leading to an increased in the secretion of soluble CD14, and other pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and hsCRP). We have recently demonstrated that plasma levels of sCD14, IL-1β, IL-6, TNF-α, and hsCRP are significantly higher among virally suppressed Kenyan PWH compared to HIV-negative participants implying that microbial translocation and inflammation persist after ART (**Table 1**).[4, 5, 20] More importantly, we were able to show for the first time positive association between sCD14 and markers of vascular inflammation (soluble VCAM-1 and ICAM-1) among African PWH further suggesting the role of microbial translocation towards vascular dysfunction in HIV.[20]

Table 1: Comparison of soluble biomarkers of gut barrier disruption, and Inflammation among our study participants

	HIV-	PWH	P value*
	(N= 266)	(N=275)	
hsCRP, mg/mL	1.8 (3.6)	2 (3.7)	0.04
I-FABP, pg/mL	2091 (2007)	3491 (2703)	0.02
sCD14, ng/ml	1690 (1614)	2668 (2164)	0.001

Data is shown as median (interquartile range); * Wilcoxon rank sum tests[4, 5]

A3. HIV is associated with an altered T-cell function and may promote hypertension independent of the circulating inflammatory mediators. Chronic immune activation of T cells and B cells was identified as a hallmark of HIV infection early in the AIDS epidemic. This is still the case even after ART treatment as noted in a few studies from US and Europe.[21, 22] Whether virally suppressed African PWH display exaggerated T cell activation after ART is yet to be known. Activated lymphocytes are now increasingly recognized as a major contributor to the pathogenesis of hypertension in uninfected persons[17]. Mice lacking both T and B cells have been reported to develop blunted HTN after challenge with salt.[3] Hypertensive adults have been reported to have significantly higher frequency of proinflammatory CD8+ T cells and cytokines indicative of T cell activation including IL-17A compared to the non-hypertensive persons.[23] To our knowledge, no previous studies have directly evaluated the role of activated lymphocytes on hypertension among PWH on stable ART. This is the overall goal of this application.

B. INNOVATION

The innovation is the **focus on understanding the immunopathogenesis of HTN in HIV** with the goal of identifying mechanistically novel pathways that would aide in the development of new therapeutic treatments for hypertension and health outcomes of PWH by preventing cardiovascular disease. Other innovations include:
1) The unique dataset of a well-characterized large cohort of African PWH as well as HIV negative controls.
2) Investigation of the interaction between latent tuberculosis, HIV and hypertension, on which almost no published data exist and which have been neglected in the hypertension studies in high-income countries.
3) Inclusion of women in studies, as North American and European cohorts are heavily skewed to men.
4) The low rates of hypertension diagnosis and treatment (2%) among our cohort will allow for the assessment of the natural history of blood pressure (BP).
5) Targeting immune system as a new strategy to reduce hypertension.
6) Inclusion of HIV negative as well as PWH when evaluating inflammation and hypertension has not been previously done in African studies.

C. APPROACH

C.1. Overall Strategy: We will leverage on clinical data and cryopreserved samples (plasma and peripheral blood mononuclear cells (PBMC) from K01 funded study (data and samples were collected between November 2018 and September 2019) among 263 PWH on stable ART and 214 age-sex matched uninfected controls in western Kenya. This proposed study can be conducted using this *CFAR NIA award* mechanism because of the significant other resources being leveraged from Dr. Temu K01 award. The amount awarded will enabled us to embark on some analysis for **Aim 1a** (cytokine assays) but will not completely fund the aim and will not fund any of the proposed laboratory work for Aim 1b. Therefore, we will use a portion of the budget for this application to cover the cost of cytokine assays as described in Aim 1a and T-cell activation **Aim 1b** (Table 2).

Hypothesized Events	Measurements Proposed
HIV ↓ Gut Barrier dysfunction	Serum I-FABP
↓ Microbial Translocation	Serum LBP and β-glucan
↓ Immune Activation	*Innate immune system:* serum cytokines: hsCRP, IL-6, sCD14, IL-1β, TNF-α *Adaptive immune system:* - Serum cytokines: IL-4, IFN-γ, IL-5, IL-17, IL-10 - Lymphocyte surface markers: ▪ Cell subset: CD3, CD4, CD8, ANK44, ANK61 ▪ Activation: CD38, HLA-DR, PD-1
Hypertension	Blood pressure >140/85

Table 2. Left column, hypothesized events leading to microbial translocation, inflammation, and hypertension. Right column proposed measurements to test the hypothesized pathways to hypertension in HIV.

Ethics: The parent study was approved by the Institutional Review Board at UW and Kenyatta National Hospital Ethical Review Committee.

Participant enrollment: PWH were recruited from Kisumu District Hospital (KDH) Comprehensive Care Center (CCC), a HIV clinic that reports 12,903 registered African patients with a broad mix of urban middle class, urban poor, and rural populations. HIV prevalence is Kisumu County is 19.1%[24]. HIV negative were recruited from community outreach to family members attending clinic at KDH CCC and had to test negative for HIV at the time of enrollment. All participants were between age 30 to 70 years of age. PWH had to be on ART for >6 months. **Exclusion criteria for all subjects** included pregnant women, those with diagnosis related to CVD, acute illness in the past month, and those currently on metformin, statins or steroids. We further excluded those with history of bowel resection, and those on deworming pills or antibiotics other than cotrimoxazole in the past 3 months.

Table 3: Study procedures covered by the parent and K01 grant		Proposed analysis to be covered by CFAR award
Questionnaire: Demographics, height, medications, weight, and BP	x	
Serum and Plasma preparation: Fasting blood lipid profile and glucose, CD4 count, viral load, and cytokine assays	x	Lipopolysaccharides, beta-glucan, and cytokine assays
PBMCs collection	X	T cell activation markers
Latent TB assay	X	

Clinical procedures: Participants completed a questionnaire about; HIV disease history (for the PWH), sexual behavior, medication exposure, diet, alcohol intake, and medical history. Weight height, waist and hip circumference, and blood pressure were also measured.

Laboratory studies: 25 ml fasting blood sample was collected, processed for plasma and PBMC and stored for future testing. Stool samples were also collected for future use.

C.2. Design and Laboratory Methods Relevant to Specific Aims 1-2

Overview: To assess whether a relationship exists between HIV, systemic immune activation, and hypertension, we will measure and compare plasma and cellular markers of systemic immune activation in these four sub-groups as shown in **Table 4**. Since tuberculosis infection rates are high among PWH in Kenya (33%), we will control for these co-infections[8]. Of the 250 PWH enrolled in the parent study, 83 were found to have hypertension. The mean (SD) age is 47±12 years and 50% are female. Mean CD4 T-cell count is 506 cells/mm^3, and all had undetectable viral load (HIV RNA <50 copies/mL).

Group	HIV Status	Hypertension	N
1	Positive	Y	83
2	Positive	N	176
3	Negative	Y	83
4	Negative	N	83

Table 4. Patient Groups

Aim 1a: Innate immune activation markers: We will measure plasma levels of selected biomarkers for gut epithelial disruption (intestinal fatty acid binding protein, I-FABP), microbial translocation (β-glucan and lipoprotein binding protein [LBP]), and innate immune activation (sCD14, IL-1β, IL-6, and TNF-α) implicated in CVD as shown in Table 2 using ELISA, and hsCRP using a Cobas Analyzer (Roche) at Dr Stephen Polyak laboratory at UW, Seattle *(Dr. Temu will perform these assays and has experience running similar assays*). The primary biomarker for microbial translocation will be plasma LBP. Some other studies have used LPS but it has poor reproducibility, with reported inter-assay variability ranging from 25 to 30%, compared to that of LBP (6%).[25] The primary endpoint for Aim 1a and 2a will be plasma IL-6, rather than hsCRP since our preliminary data demonstrating stronger associations of IL-6 levels than hsCRP with blood pressure.[26]

Aim 1b: Adaptive immune activation markers: We propose to measure a range of biomarkers related to cell-specific and global T cell immune activation. PBMCs will be stained and analyzed on a FACS18-color SLR flow cytometer at the UW CFAR Immunology Core laboratory. The surface staining panel will be as follows: CD3, CD4, CD8, CD14, CD16, CD19, CD56 and fixable live-dead aqua. We will look at activated CD4+ and CD8+ T-cell subsets, using the mean percentage of activated CD38$^+$HLA-DR$^+$ as our **primary outcome endpoint for Aim 1b and 2b**. Secondary outcomes will include IL-17A, a plasma marker of Th17 cell activation reported to be associated with hypertension in the general population.[23] In addition, we will measure plasma cytokines indicative of Th1, Th2, and Th17 immune responses using ELISA based assays. Our research group frequently ships PBMC from Kisumu CDC laboratory to Seattle, we typically recovery between 8-9.4x10^5 viable cells/cryopreserved vial at 1x10^6 cells/vial after 1-2 years of storage in liquid nitrogen. Therefore, even though there is a potential to lose some cells, we should have sufficient amount given that only about 6x10^6 viable cells per participants are needed for this analysis. Studies have also demonstrated that flow cytometry assay results for cryopreserved PBMC (up to 24 months) and fresh PBMC are highly correlated.[27]

C.3. Statistical Analysis Plan and Sample Size Calculation:

Sample size: In our previous study, the mean and standard deviation for IL-6 was were 1.41 and 0.65 respectively among 277 PWH[5]. Based on these data, if we compare 249 PWH (required for **Aim1**) versus the 176 uninfected controls, we will be able to detect a minimum detectable difference in plasma IL-6 levels of approximately 0.25 µg/ml with 80% power and a 2-sided significance level of 0.05. The spearman correlation coefficient between IL-17A and hypertension was reported at 0.25 from a small study conducted in the US general population. Based on these data for **Aim 2**, the 245 PWH will give us greater than 80% power to detect significant association between IL-17A and hypertension.

Aim 1: We will compare PWH and uninfected controls using the non-parametric Wilcoxon rank-sum test or linear regression with group assignment as an independent variable. Correlates of inflammation and immune activation will be evaluated using generalized regression models. Predictors will include HIV status, age, sex, cardiovascular risk factors (body mass index smoking status, fasting glucose, latent tuberculosis infection, and total, LDL and HDL cholesterol levels). For all participants. For PWH we will also evaluate CD4 nadir, CD4 current, and duration of ART as potential predictors of systemic inflammation. The association between each biomarker and other covariates will be studied using linear regression analysis.

Aim 2: The primary analyses for Aim 2 will use linear regression with IL-6 as the primary independent variable. In addition, we will also use logistic regression (Aim 2) to estimate the association between immune activation and hypertension prevalence. We will divide the biomarkers into quartiles (highest quartile [>75th percentile] vs. lower three quartiles). We plan to define elevated biomarkers as levels >75% because with the exception of hsCRP it is still unclear what thresholds are associated with clinical events for these biomarkers. This is also consistent with other studies that have looked at the association between biomarkers with health risks using biomarker quartiles[18, 28, 29]. With 415 subjects for Aim 1, and 249 subjects for Aim 2, each regression model will be able to include large number of covariates. The PI will be assisted with these analyses by Barbra Richardson (Professor of Biostatistics-*see letter in appendix*).

C.4. Expected Outcomes and alternative approaches: Based on our hypothesis, we anticipate that HTN will be associated with increased markers of immune activation and inflammation as measured in Aim 1. The large sample size of our study gives us enough power to detect small but significant differences between t groups. A third arm of ART naïve PWH would have been informative and was considered but was not adopted because it was not feasible to recruit 245 ART-naïve participants. Kenya like many other African countries have rolled out test and treat guideline therefore all patients regardless of the CD4+ count are initiated on ART at the time of diagnosis.

D. CONCLUSIONS AND FUTURE DIRECTIONS

The proposed studies will identify immune factors potentially suitable as surrogate endpoints for future intervention trials, and increase our understanding of pathogenesis of hypertension, a subject with broad relevance to both HIV positive and the uninfected populations. Further research directions that could be pursued include:

If we determine that there is an association between immune activation and hypertension, it may be of interest to initiate a longitudinal study to investigate how changes in markers of inflammation, would predict risk for incident hypertension in this population. Careful research documenting immunologic change during ART will allow us to make conclusions regarding the role of immune activation in predicting risk for CVD in PWH. We also plan to use the stored stool and plasma to assess the contribution of the altered microbiome and microbial metabolite signaling towards hypertension.

Table 5: Research Plan Schedule	**Funding Period**		
	Pre-Award	**Year 1**	**Year 2**
Human Subjects Approval	Pending		
Aim 1a - Cytokine Assays		**X**	
Aim 1b - Cellular immune activation markers		**X**	
Data analysis & manuscript preparation			**X**
Aim 2 - Data analysis & manuscript preparation		**X**	**X**

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