Correlates and outcomes of high inflammatory cytokine levels in ART-naïve Kenyan children diagnosed with HIV at hospital admission

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Abstract

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Background:

Cytokines are the key mediators of innate and adaptive immunity and the key modulators of inflammation during acute illness. Owing to the immune dysregulation and inflammation during HIV-infection, multiple studies have reported the association of inflammatory cytokines with increased mortality and other adverse clinical outcomes in people living with HIV (PLH) regardless of antiretroviral therapy (ART) status. However, very few studies have investigated the predictors of inflammatory cytokines and their association with adverse clinical outcomes in children living with HIV (CLHIV). The objective of our study was to identify the correlates of

high proinflammatory cytokine levels at hospital admission and whether high cytokine levels were associated with an increased risk of death and prolonged hospitalization among newly diagnosed hospitalized CLHIV.

Methods:

Cytokine levels were measured in repository serum specimens from a prior randomized clinical trial of hospitalized Kenyan CLHIV, who were diagnosed with HIV infection at hospital admission, and randomized to initiate ART either urgently or after medical stabilization. Based on the distribution of cytokine levels, the cytokines were treated as either binary (detected/undetected) (IL-1β, Il-2, TNF-α, IFN-γ, IL-4, IL-10, IL-13) or continuous (IL-6, IL-8, IL-12p70) for the analyses. Cytokines IL-1β, IL-2, IL-6, and TNF-α were selected as primary cytokines of interest given their association with mortality in the critical care literature. Primary study endpoints were defined *a priori* as death within six months of hospital admission, the combined end point of death or continued hospitalization at 15 days, and the mean duration of hospitalization. The potential correlates of cytokines were selected from existing literature and assessed using Poisson regression models for binary outcomes and linear regression models for continuous outcomes. Linear regression was used to assess the association between cytokine levels and duration of hospitalization in survivors. Cox regression and Kaplan-Meier survival analysis were used to estimate 6-month hazard ratios and median time to death. Poisson regression was used to estimate the relative risk of the combined endpoint. All analyses were adjusted for age and sex at admission.

Results:

Of 181 CLHIV in the overall RCT, a total of 104 children had sufficient enrollment serum for cytokine testing. IL-6 and IL-8 were detected in nearly all children (103 for each cytokine, 99.04%) children while IL-1β was detectable in only 7 children (6.31%). After adjusting for age and sex at enrollment, higher baseline C-reactive protein (CRP) ([adjusted coefficient (aCoefficient)] = 0.008 [95% CI = 0.00, 0.001, p=0.04]), higher CD4% (aCoefficient = 0.04 [95% CI = 0.00, 0.08, p=0.029]) and lower CD4% <15 (aCoefficient = -0.86 [95% CI = -1.59, -0.14] p=0.02]) were associated with higher IL-6 levels. Total neutrophil count ([adjusted Prevalence Ration (PR)] = 0.82 [95% CI = 0.72, 0.94], p=0.004) and hemoglobin level (g/dL) ([aPR] = 0.84 [95% CI = 0.72, 0.98], p=0.027) were independently associated with decreased IL-2 detection. Higher $\log_{10}$ CMV DNA levels (adjusted prevalence ratio [aPR] = 8.50 [95% CI = 1.99, 36.25], p=0.004) were independently associated with increased IL-1β detection. Meanwhile, the recruitment site (Kisumu) ([aPR] = 0.12 [95% CI = 0.015, 0.97], p=0.047) and administration of steroid at enrollment ([aPR] = 3.97e-07 [95% CI = 1.20e-07, 1.32e-06], p<0.01) were significantly associated with decreased IL-1β detection in the adjusted analysis. We did not identify any significant correlates of TNF-α. In adjusted analyses, each doubling of IL-6 levels (1-$\log_2$ increase) was associated with a 27% increased hazard of death ([aHR] = 1.27, [95% CI = 1.07, 1.50]). None of the other cytokines were associated with 6-month mortality risk or the combined outcome. Additionally, there was no association between any cytokines and the duration of hospitalization.

Conclusion:

IL-2 and Inflammatory cytokines were detected at high levels in this cohort of hospitalized newly diagnosed CLHIV. IL-1β detection was positively associated with CMV DNA levels and

was negatively associated with the receipt of steroids IL-6 levels were associated with biomarkers of inflammation (CRP), and severe immunosuppression, and higher IL-6 level was independently associated with an increased hazard of death over 6-months in ART-naive Kenyan CLHIV diagnosed at hospital admission. Our study provides some novel insights into the immunological status and prognosis of severely ill, ART-naïve CLHIV, highlighting the importance of inflammation in critical care outcomes in this population.

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ABSTRACT:

Background: Cytokines are the key mediators of innate and adaptive immunity and the key modulators of inflammation during acute illness. Inflammatory cytokines are associated with increased mortality and other adverse clinical outcomes in people living with HIV (PLH) regardless of antiretroviral therapy (ART) status. However, few studies have investigated the predictors of inflammatory cytokines and their association with adverse clinical outcomes in children living with HIV (CLHIV). The objective of our study was to identify the correlates of high proinflammatory cytokine levels at hospital admission and whether high cytokine levels were associated with an increased risk of death and prolonged hospitalization.

Methods: Cytokine levels were measured in repository serum specimens from a prior randomized clinical trial (RCT) of hospitalized Kenyan CLHIV, who were diagnosed with HIV-infection at hospital admission and randomized to initiate ART either urgently or after medical stabilization. Cytokines IL-1β, IL-2, IL-6, and TNF-α were selected as primary cytokines of interest given their association with mortality in critical care literature. Primary study endpoints were defined *a priori* as death within six months of hospital admission, the combined end point of death or continued hospitalization at 15 days, and the mean duration of hospitalization. Potential correlates of cytokines were selected from existing literature and assessed using Poisson regression models for binary outcomes and linear regression models for continuous. Linear regression was used to assess the association between cytokine levels and duration of hospitalization in survivors. Cox regression and Kaplan-Meier survival analysis were used to estimate 6-month hazard ratios and median time to death. Poisson regression was used to estimate the relative risk of the combined endpoint. All analyses were adjusted for age and sex at admission.

Results: Of 181 CLHIV in the overall RCT, 104 (57.4%) had sufficient enrollment serum for cytokine testing. IL-6 and IL-8 were detected in nearly all children (103 for each cytokine, 99.04%) children while IL-1β was detectable in only 7 children (6.31%). After adjusting for age and sex at enrollment, higher baseline C-reactive protein (CRP) ([adjusted coefficient (aCoefficient)] = 0.008 [95% CI = 0.00, 0.001, p=0.04]), higher CD4% (aCoefficient = 0.04 [95% CI = 0.00, 0.08, p=0.029]) and lower CD4% <15 (aCoefficient = -0.86 [95% CI = -1.59, -0.14] p=0.02]) were associated with higher IL-6 levels. Total neutrophil count ([adjusted Prevalence Ration (PR)] = 0.82 [95% CI = 0.72, 0.94], p=0.004) and hemoglobin level (g/dL) ([aPR] = 0.84 [95% CI = 0.72, 0.98], p=0.027) were independently associated with decreased IL-2 detection. Higher $\log_{10}$ CMV DNA levels (adjusted prevalence ratio [aPR] = 8.50 [95% CI = 1.99, 36.25], p=0.004) were independently associated with increased IL-1β detection. Meanwhile, the recruitment site (Kisumu) ([aPR] = 0.12 [95% CI = 0.015, 0.97], p=0.047) and administration of steroid at enrollment ([aPR] = 3.97e-07 [95% CI = 1.20e-07, 1.32e-06], p<0.01) were significantly associated with decreased IL-1β detection in the adjusted analysis. We did not identify any significant correlates of TNF-α. In adjusted analyses, each doubling of IL-6 levels (1-$\log_2$ increase) was associated with a 27% increased hazard of death ([aHR] = 1.27, [95% CI = 1.07, 1.50]). None of the other cytokines were associated with 6-month mortality risk or the combined outcome. Additionally, there was no association between any cytokines and the duration of hospitalization.

Conclusion: IL-2 and Inflammatory cytokines were detected at high levels in this cohort of hospitalized newly diagnosed CLHIV. IL-1β detection was positively associated with CMV DNA

levels and was negatively associated with the receipt of steroids IL-6 levels were associated with biomarkers of inflammation (CRP), and severe immunosuppression, and higher IL-6 level was independently associated with an increased hazard of death over 6-months in ART-naive Kenyan CLHIV diagnosed at hospital admission. Our study provides some novel insights into the immunological status and prognosis of severely ill, ART-naïve CLHIV, highlighting the importance of inflammation in critical care outcomes in this population.

Keywords: HIV, herpesviruses, cytokines, CMV, EBV, mortality, interleukin-6 (IL-6), inflammation

INTRODUCTION:

HIV infection leads to persistent inflammation and immune activation leading to adverse clinical outcomes and mortality (1,2). Cytokines are the key mediators of innate and adaptive immunity and the key modulators of inflammation (3). In response to invading pathogens, cells produce cytokines with diverse functions that modulate the immune response and clear infection. Through the course of an infection, cytokines assist with inflammation, cellular activation, proliferation, and apoptosis. As infection is controlled or cleared, cytokines help downregulate the immune response to prevent further damage to the host (4). A cascade of inflammatory responses begins after HIV-infection, prompting the production of pro- and anti-inflammatory cytokines leading to a persistent inflammatory state. Because of the vigorous response to HIV infection, compared to HUI (HIV-uninfected individuals) and people living with HIV (PLH) typically have elevated levels of most antiviral and inflammatory cytokines (5–7). Once antiretroviral therapy (ART) is initiated, and HIV viral levels are brought under control, most inflammatory and antiviral cytokine levels decrease (8–10). However, even following successful ART, pro-inflammatory cytokine levels (including TNF-α, IL-4, IL-6, IL-8, and IL-10) remain elevated in PLW compared to HIV-uninfected individuals, which is a result of residual immune dysfunction not completely restored by ART (5–7).

Higher levels of pro-inflammatory cytokines have been found to be associated with an increased risk of death in hospitalized people with advanced HIV (11–14). In a Brazilian cohort of hospitalized PLH >12 years of age -- with both ART-naive and ART-initiated individuals --higher IL-8 was associated with an increased risk of death (13). Higher IL-6 levels have been consistently associated with increased mortality risk in the critical care setting (15) and was associated with mortality in ART-naïve adults in two independent studies based in Rakai, Uganda, and Benin City,

Nigeria respectively (16,17). Other biomarkers including C-reactive protein (CRP) and D-dimer, have also been associated with mortality in both ART-naive and ART-initiated PLH (18,19). Meanwhile, in early ART-era studies, IL-10 and TNF-α have been associated with mortality in PLH (20,21).

While many studies have found higher cytokine levels in PLWH, and associations between high inflammatory cytokines and death, to date there is sparse data on children living with HIV (CLHIV) in the critical care setting (22–28). Despite prompt treatment and initiation of ART, hospitalized CHLIV still have extremely high mortality, and novel interventions are needed to improve outcomes and identify the children at the highest risk of death. We conducted a retrospective study using specimens and data from a randomized controlled trial of CLHIV diagnosed with HIV infection during hospital admission, who were randomized to either urgent (within 48 hours) or post-stabilization ART (within 14 days) and followed for six months (29). We investigated correlates of high proinflammatory cytokine levels at hospital admission and determined whether high cytokine levels were associated with an increased risk of poor clinical outcomes.

METHODS

Study setting

This is a retrospective cohort study nested within an existing cohort. The PUSH Study (NCT02063880) was an unblinded randomized clinical trial of Kenyan ART-naïve CLH ≤ 12 years of age enrolled at hospital admission (29). The participants were randomized to receive urgent (<48 hours) versus standard antiretroviral therapy (ART) (initiated post-stabilization at 7-14 days). Primary outcomes were 6-month all-cause mortality rate, the incidence of immune reconstitution

inflammatory syndrome (IRIS), and the incidence of drug toxicity in the urgent versus early ART children. The clinical sites were Kenyan hospitals in Kisumu and Nairobi, Kenya. Additional study details have been previously published elsewhere (29,30). Out of the 181 children enrolled, randomized, and followed-up in the parent study, all children with sufficient serum enrollment samples available for cytokine assessments were included in this nested cohort (n=104, Figure 1). The parent RCT found no difference in mortality between randomization arms.

Cytokine assays

Cytokine levels were measured from frozen serum using the V-PLEX Proinflammatory Panel 1 Human Kit as per manufacturer instructions (Meso Scale Diagnostics, Maryland, USA). The assay quantifies ten biomarkers associated with inflammatory response and immune system regulation (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α). The cytokines IL-6, IL-2, TNF-α, and IL-1β were assessed in the primary analysis, given previous associations with mortality, and co-infections in both PLH and CLHIV (11,17,23,31–35). The remaining six cytokines were of less interest and treated as secondary analyses. Based on the distribution of cytokine levels, the cytokines were treated as either binary (detected/undetected) (IL-1β, Il-2, TNF-α, IFN-γ, IL-4, IL-10, IL-13) or continuous (IL-6, IL-8, IL-12p70) for the analyses. Since the IL-6, IL-8, and IL-12p70 distribution was highly skewed to the right, values were $\log_2$-transformed to normalize the data, such that each 1 log increase is interpreted as a doubling of cytokine level. A priori, it was decided that cytokines with >30% undetectable values would be treated as detectable/undetectable variables.

Statistical analysis

Correlates of high cytokine levels, or detectable cytokines in serum:

Potential correlates of detectable or high levels of proinflammatory cytokine levels were selected based on a hypothesized relationship with inflammation, or evidence from existing literature. We included demographic factors (age, Kisumu or Nairobi recruitment site), nutrition status (WHO Z-scores, severe acute malnutrition), immune status (WHO stages, severe immunosuppression, steroid prescription at enrollment), laboratory/clinical status (total neutrophil count, CRP, CD4%, hemoglobin levels, HIV RNA), and herpesvirus viremia (Cytomegalovirus (CMV) and Epstein-Barr virus (EBV)) (22,27,30,36–38).

The demographic factors and medical history were collected at hospital admission and clinical information regarding hospitalization, treatments, and outcomes were collected at 2 weeks, and monthly following start of ART, until 6 months. Severe immunosuppression was defined using age and CD4% or count according to WHO criteria (<12 months, <30%; 12-35 months, <25%; 36-59 months, <20%; over 5 years, <350 cells/μl). Meanwhile, plasma CMV and EBV levels were obtained through real-time quantitative PCR with the limit of detection for both CMV and EBV at 1 copy per reaction, as previously described (30,39). Since levels $\geq$ 1000/ml are associated with clinical outcomes in the critical care setting, the 1000/ml cutoff was used for a categorical definition of CMV and EBV viremia. Poisson regression was used to estimate prevalence ratios for binary outcomes (IL-1β, Il-2, TNF-α, IFN-γ, IL-4, IL-10, IL-13), and linear regression was used for continuous outcomes (IL-6, IL-8, IL-12p70). Each cytokine variable was assessed individually and then adjusted for confounders, including age at enrollment and sex assigned at birth.

Clinical outcomes:

The primary study endpoints were defined a priori as death within six months of hospital admission, the combined end point of death or continued hospitalization at 15 days, and the median duration of hospitalization. A detailed summary of predictors of mortality is presented elsewhere (30). The primary exposures were cytokine detection (binary) and cytokine levels (continuous $\log_2$ transformed ng/ml). Linear regression was used to assess the association between cytokine levels and duration of hospitalization in survivors. Cox regression and Kaplan-Meier survival analysis were used to estimate 6-month hazard ratios and median time to death. Poisson regression was used to estimate the relative risk of the combined endpoint. All analyses were adjusted for age and sex at admission. All statistical analyses were conducted using STATA v.17 (StataCorp, College Station, Texas, USA) and R (2022.12.0 + 353).

RESULTS

Population characteristics

Out of the 181 children randomized, enrolled, and followed up in the parent PUSH cohort, 104 (57.4%) children had adequate serum for cytokine testing and were included in this nested study (Table 1). The median age range in years was 2.05 (IQR: 0.99, 6.02) out of which 51 (49.0%) were under the age of two. A total of 24 (23.0%) children died before 6 months, 44 (42.3%) met the combined endpoint, and the median duration of hospitalization was 11 (IQR: 6, 17) days.

The detailed baseline characteristics of the cohort including demographic factors, nutrition status, immune status, laboratory/clinical status, and key outcomes are presented in Table 1. A total of 28.8% (21/73) of children had severe acute malnutrition (SAM) at enrollment, 69.2% (72/104) had pneumonia with 24.6% (15/61) being diagnosed with pneumonia with hypoxia, and

71.8% (74/103) were at WHO stage III/IV. Out of the 66 children who were tested for CMV and EBV, 37 (56.1%) were CMV viremic at enrollment, 48 (72.3%) for EBV, and 28 (42.4%) had both EBV and CMV viremia. A total of by 82 (79.6%) children had severe immunosuppression and 58 (55.77%) had CD4% < 15%. The median $\log_{10}$ HIV RNA copies/ml was 5.76 (5.15, 6.27) copies/ml.

Cytokine levels in the cohort

Nearly all cytokines were significantly elevated in the PUSH Cohort, compared to a healthy birth cohort of HIV-uninfected Kenyan children in a similar age range (Supplemental Figure 1). Table 2 provides the mean cytokine levels, and proportion of children with detectable cytokines of interest. Both IL-6 and IL-8 were detected in nearly all children (103, (99.04%), so these were treated as continuous variables only. IL-1β was detectable in only 7 (6.31%) children. Approximately 29% of children had undetectable IL-12p70, so this cytokine was treated as both continuous and binary (detectable/undetectable).

Correlates of high cytokine levels at enrollment

Correlates of cytokines at enrollment are provided in Table 3. Higher $\log_{10}$ CMV DNA levels (adjusted prevalence ratio [aPR] = 8.50 [95% CI = 1.99, 36.25], p=0.004) were independently associated with increased IL-1β detection. Meanwhile, the recruitment site (Kisumu) ([aPR] = 0.12 [95% CI = 0.015, 0.97], p=0.047) and administration of steroid at enrollment ([aPR] = 3.97e-07 [95% CI = 1.20e-07, 1.32e-06], p<0.01) were significantly associated with decreased IL-1β detection in the adjusted analysis. Total neutrophil count ([aPR] = 0.82 [95% CI = 0.72, 0.94], p=0.004), hemoglobin level (g/dL) ([aPR] = 0.84 [95% CI = 0.72,

0.98], p=0.027), and CD4% ([aPR] = 0.96 [95% CI = 0.92, 1.00], p=0.04) were independently associated with decreased IL-2 detection (Table 3). CMV ≥1000 copies/ml was significantly associated with increased IL-2 detection in univariate analysis but did not retain significance after adjustment. Higher C-reactive protein (CRP) (aCoefficient = 0.008 [95% CI = 0.00, 0.001, p=0.04]) and higher CD4% (aCoefficient = 0.04 [95% CI = 0.00, 0.08, p=0.03]) were associated independently with higher IL-6 levels. Meanwhile, CD4% <15% (aCoefficient = -0.86 [95% CI = -1.59, -0.14] p=0.02]) was associated with lower IL-6 levels. We did not identify any significant correlates of TNF-α. The correlates of the cytokines of secondary interest are provided in Supplementary Table 2. None of the covariates retained significance after adjustment for sex and age at enrollment.

High cytokine levels and clinical outcomes

The Kaplan-Meier survival curves for the primary cytokines are presented in Figure 2 and point estimates for associations between cytokines and the three clinical outcomes are provided in Table 4. After adjusting for age and sex at enrollment, each doubling of IL-6 levels was associated with a 27% increased hazard of death (adjusted hazard ratio [aHR] = 1.27, [95% CI = 1.07, 1.50]). IL-6 levels were not associated with the combined outcome of death or continued hospitalization at 15 days. None of the other cytokines were associated with death or the combined outcome of death or continued hospitalization at 15 days. Additionally, there was no association between any cytokine and the duration of hospitalization. Data for secondary cytokines and clinical outcomes are provided in Supplemental Table 4.

DISCUSSION

In this study of Kenyan CLHIV who were diagnosed with HIV infection and started ART shortly after hospital admission, we found high levels of inflammatory cytokines compared to healthy children from the community. Children with higher neutrophils, higher hemoglobin, and CMV viremia were more likely to have detectable IL-2 levels. Higher CMV DNA level was independently associated with IL-1β detection. Children with CD4% less than 15% had lower IL-6 levels. Higher IL-6 levels were associated with an increased risk of 6-month mortality.

IL-6 is an important mediator of acute inflammation, and our finding that IL-6 was associated with other markers of inflammation, CD4 percentage, and immunosuppression is consistent with other studies demonstrating that IL-6 is elevated and associated with worse prognosis in hospitalized cohorts. In the PUSH cohort, each doubling of IL-6 level at hospital admission was associated with a 27% increased hazard of death. IL-6 is a mediator of acute inflammation and is elevated during severe infections, including pneumonia and severe sepsis (23,25,31,35,40,41). Higher IL-6 levels have consistently been associated with increased risk of mortality in adults and children in critical care settings (11,16,31,42). In outpatient studies of PLWHIV, multiple studies have also found an association between increased IL-6 levels with an increased risk of AIDS-related and non-AIDS-related mortality and other adverse clinical outcomes including neuropsychiatric outcomes, anemia, cancer, and cardiovascular disease (32). As blood IL-6 levels are elevated in PLH regardless of ART status, multiple studies have reported a strong association between IL-6 levels and mortality (16,31,35,43). IL-6 levels were independent predictors of death in both ART-naive PLH (adjusted odds ratio [aOR] = 3.8, [95% CI: 1.8–7.8] and ART-initiated PLH ([aHR] = 2.34, [95% CI = 1.51, 3.63]) (11,12,44).

We found associations with IL-2 that are consistent with what we know about cytokine biology, and with other research studies. IL-2 plays an important role in cellular proliferation and differentiation. Each unit increase in total neutrophil count, hemoglobin levels (g/dL), and CD4% at enrollment was associated with 18%,16%, and 4% less likelihood of IL-2 detection, respectively. The co-occurrence of neutropenia and anemia in PLH has been reported extensively (45–47). Chronic HIV-infection has been found to initiate immunosuppressive activity of neutrophils which in turn inhibits T-cell function impacting hematopoietic processes and causing immune dysregulation resulting in abnormal cytokines expression -- marked by significantly low IL-2 levels and elevated TNF-α levels in PLH (45,48,49). On the other hand, a study in severely anemic CLHIV without blood transfusion found no correlations between the proportion of either dyserythropoietic or apoptotic cells and the peripheral blood concentrations of cytokines TNF-α (50) which was consistent with the results from our study. Additionally, IL-2 dysregulation has been reported to be associated with depleting CD4+ lymphocytes during chronic HIV-infection through unrevealed pathways which is consistent with our findings (51). Additional scientific endeavors are required to assess the association of TNF-α with anemia particularly in hospitalized CLHIV.

The herpesvirus CMV was also found to be associated with increased detection of IL-1β which are produced by activated monocytes and macrophages and has pleiotropic effects on the immune response. CMV commonly reactivates in critical care settings in both immunocompetent (36) and immunocompromised (30) populations and has been associated with increased risk of mortality in adults (52) and children (39). In the PUSH cohort, we previously reported CMV viremia $\geq$1000 IU/ml at admission was associated with an increased risk of the combined outcome of death or continued hospitalization at 15 days, and a longer duration of hospitalization (30).

CMV replication has been reported to increase production of several inflammatory cytokines *in vitro* and *in vivo*, including TNF–α, IL–6, and IL-8 (53,54). CMV has also been known to be an inducer of IL-1β leading to disruption of the intestinal epithelial barrier (54–56). Maidji et. al. reported in a cohort of PLH and PLWH, that CMV increased the production of IL-6, but not TNF-α or IL-1β *in vitro* (54). Furthermore, in a mostly (93.8%) CMV viremic cohort of PLH adults on ART, the authors reported no differences in IL-1β cytokine responses between CMV-seropositive versus -seronegative individuals (55). Meanwhile, in this study, we found IL-1β, while sparsely detected in the cohort, was 8.5-fold more likely to be detected with each 1-log increase in CMV DNA level. Further research is necessary to delineate a better understanding of the association between CMV and IL-1β. We also found that IL-1β was less likely to be detected in CLHIV who were on steroid at enrollment. This is consistent with existing literature as the administration of corticosteroids has been reported to inhibit HIV-related immune activation (57). However, the evidence of its impact on immunologic profile of PLH with advanced disease has been sparse and needs further investigation (58).

While our study has many strengths, we were limited by the availability of all participant specimens from the parent PUSH study for cytokine analysis as the specimens had been used for various assays previously and lowered our statistical power. As we assessed cytokine levels in serum at baseline, it may not accurately reflect cytokine levels in other body regions. Also, the strength of the association of baseline cytokine levels with mortality and adverse clinical outcomes may be lower when compared to the levels later in clinical decline (18).(19) Although we assessed the cytokines levels as either binary (detected/not detected) or continuous in this study, there are no universally accepted empiric cytokine levels for clinical significance. Hence, simply using detected/not detected or continuous levels of these biomarkers may not accurately reflect

biological levels sufficient to exert inflammation and host damage. While it appears that higher cytokine levels may be associated with increased mortality from our KM Survival analysis, with a small absolute number of deaths (n=24), we had low statistical power to detect small effect sizes. Finally, we did not assess other known biomarkers of mortality in CLHIV including D-dimer and soluble CD14 among others (11,18,42).

Our study provides some novel insights into the immunological status and prognosis of severely ill, ART-naïve CLHIV. Cytokine levels were universally elevated but were unexpectedly not associated with HIV levels. Plasma CMV levels were found to predict a much higher risk of IL-1β detection, and this should be explored in future studies. Consistent with previous research, we found that IL-6 was independently associated with increased host inflammation and immunosuppression, and predicted a higher risk of mortality, highlighting the importance of inflammation in critical care outcomes in this population.

TABLES AND FIGURES:

Table 1: Participant Characteristics.

Characteristic	N	Median IQR / n (%)
Age at enrollment (years)	104	2.05 (0.99, 6.02)
Age under 1	104	26 (25.00)
Age under 2	104	51 (49.04)
Age 2 to 5 years	104	23 (22.12)
Age 5 to 12 years	104	30 (28.85)
Sex at enrollment, Female	104	48 (46.15)
Enrollment site, Kisumu	104	58 (55.77)
Primary caregiver mother	104	97 (93.27)
Growth indicators		
WAZ < -2 SD	99	62 (62.63)
HAZ < -2 SD	103	66 (64.08)
WHZ < -2 SD	74	33 (44.59)
Severe acute malnutrition (SAM)	73	21 (28.77)
Enrollment diagnosis and disease stage		
Pneumonia	104	72 (69.23)
Pneumonia with hypoxia	61	15 (24.59)
Suspected pulmonary TB	102	20 (19.61)
Malaria	103	22 (21.36)
Gastroenteritis	104	13 (12.50)
Persistent diarrhea	104	11 (10.58)
Baseline WHO stages III/IV	103	74 (71.84)
Herpesvirus viremia at enrollment		
CMV >=1000 copies/ml	66	13 (19.70)
EBV >=1000 copies/ml	66	31 (46.97)
Laboratory and treatment		
Total neutrophil count	99	3.17 (1.73, 5.11)
CD4%	103	12.9 (8.67, 20.91)
CD4% <15%	104	58 (55.77)
Severe immunosuppression by age and CD4	103	82 (79.61)
$\log_{10}$ HIV RNA copies/ml	95	5.76 (5.15, 6.27)
Received steroid treatment	96	6 (6.25)
Clinical outcomes		
Died before 6 months	104	24 (23.08)
Died or continued hospitalization at 15 days	104	44 (42.31)
Continued hospitalization among survivors (day 15)	80	20 (25.00)
Median duration of hospitalization (days)	95	11 (6, 17)

Table 2: Descriptive summary of cytokine detection and levels in serum of ART-naïve Kenyan children at hospital admission.

Cytokine (in serum)	**Detectable n (%)**	**Log_2 ng/mL (among detectable) Median IQR (25%, 75%)**
IL-1β	7 (6.31)	13.11 (11.37, 13.35)
IL2	26 (25.00)	10.47 (10.00, 12.73)
IL6	103 (99.04)	12.56 (11.57, 13.89)
TNF-α	47 (45.19)	9.49 (8.67, 10.64)

Table 3: Correlates of IL-1β, IL-2, IL-6, and TNF-α in serum of ART-naïve Kenyan children at hospital admission.

Factors	IL-1β detection		IL-2 detection		log_2 IL-6 level		TNF-α detection	
	Adjusted PR (95% CI)	p	Adjusted PR (95% CI)	p	Adjusted Coefficient (95% CI)	p	Adjusted PR (95% CI)	p
Age under 2	2.83 (0.36, 22.30)	0.321	1.84 (0.54, 6.28)	0.32	0.18 (-0.93, 1.31)	0.74	0.88 (0.47, 1.66)	0.71
Recruitment site: Kisumu	**0.12 (0.015, 0.97)**	**0.047**	0.71 (0.36, 1.42)	0.34	-0.16 (-0.90, 0.56)	0.65	0.92 (0.60, 1.43)	0.74
Underweight	0.71 (0.15, 3.23)	0.66	1.08 (0.55, 2.08)	0.81	0.33 (-0.44, 1.11)	0.39	1.04 (0.66, 1.63)	0.84
Wasted	0.74 (0.13, 4.32)	0.75	0.89 (0.45, 1.72)	0.73	0.36 (-0.54, 1.28)	0.43	1.15 (0.68, 1.94)	0.58
Stunted	3.54 (0.43, 28.67)	0.23	1.16 (0.56, 2.42)	0.67	0.50 (-0.24, 1.25)	0.18	1.14 (0.71, 1.83)	0.56
SAM	0.67 (0.07, 5.97)	0.72	1.04 (0.51, 2.09)	0.91	-0.20 (-1.22, 0.82)	0.69	1.45 (0.87, 2.41)	0.14
WHO Stage IV	0.49 (0.11, 2.13)	0.34	0.72 (0.38, 1.37)	0.32	0.48 (-0.31, 1.27)	0.23	1.29 (0.76, 2.19)	0.33
WHO Stage I/II	0.42 (0.17, 1.04)	0.06	0.70 (0.44, 1.10)	0.13	-0.016 (-0.55, 0.51)	0.95	1.11 (0.81, 1.53)	0.49
Severe immunosuppression	0.50 (0.12, 2.07)	0.34	4.44 (0.64, 30.79)	0.13	-0.60 (-1.52, 0.31)	0.19	0.68 (0.42, 1.10)	0.12
On steroids at enrollment	**3.97e-07 (1.20e-07, 1.32e-06)**	**0.00**	2.15 (0.87, 5.28)	0.09	-0.27 (-1.86, 1.30)	0.73	0.36 (0.06, 2.20)	0.27
Total neutrophil count	0.86 (0.69, 1.07)	0.19	**0.82 (0.72, 0.94)**	**0.004**	0.024 (-0.03, 0.08)	0.39	1.00 (0.97, 1.03)	0.82
C-reactive protein	0.97 (0.93, 1.02)	0.33	0.99 (0.98, 1.00)	0.23	**0.008 (0.00, 0.01)**	**0.04**	0.99 (0.98, 1.00)	0.25
Hemoglobin level (g/dL)	1.11 (0.88, 1.39)	0.92	**0.84 (0.72, 0.98)**	**0.03**	-0.017 (-0.20, 0.16)	0.86	0.90 (0.81, 1.00)	0.07
CD4%	1.02 (0.95, 1.10)	0.43	**0.96 (0.92, 1.00)**	**0.04**	**0.04 (0.00, 0.08)**	**0.03**	0.99 (0.97, 1.02)	0.83
CD4% <15%	0.71 (0.16, 3.12)	0.661	0.92 (0.48, 1.77)	0.81	**-0.86 (-1.59, -0.14)**	**0.02**	1.01 (0.65, 1.56)	0.95
Log10 HIV RNA copies/ml	0.56 (0.27, 1.18)	0.13	1.14 (0.72, 1.81)	0.56	-0.24 (-0.69, 0.21)	0.29	1.00 (0.76, 1.32)	0.95
CMV >=1000 copies/ml	**5.48e+07 (1.94e+07, 1.55e+08)***	**<0.001**	1.89 (0.91, 3.91)	0.08	0.74 (-0.25, 1.73)	0.14	1.39 (0.71, 2.70)	0.32
CMV (log10 copies/ml)	**8.50 (1.99. 36.25)**	**0.004**	1.14 (0.65, 2.01)	0.63	0.31 (-0.27, 0.90)	0.28	1.13 (0.80, 1.61)	0.46
EBV >=1000 copies/ml	2.98 (0.21, 42.17)	0.42	0.85 (0.37, 1.97)	0.71	0.22 (-0.55, 1.00)	0.56	1.15 (0.63, 2.10)	0.62
EBV (log10 copies/ml)	0.89 (0.21, 3.69)	0.88	0.83 (0.49, 1.39)	0.49	-0.04 (-0.50, 0.40)	0.84	1.27 (0.93, 1.72)	0.12

Notes. Only adjusted measures are provided in the table for readability. None of the associations remained significant after the Bonferroni test (correction) with four comparisons (four primary cytokines). *A total of 3 children with detectable IL-1β also had CMV>1000 copies/ml.

Table 4: Association between cytokines and clinical outcomes in Kenyan children diagnosed with HIV at hospital admission.

Outcomes	Measure of association	Cytokine exposure variable			
		IL-1β detection	**IL-2detection**	**Log_2 IL-6 level**	**TNF-α detection**
Combined endpoint	**IRR (95% CI)**	1.01 (0.41, 2.47), p=0.98	1.4 (0.88, 2.20), p=0.15	1.05 (0.94, 1.19), p=0.33	1.45 (0.92, 2.28), p=0.10
	aIRR (95% CI)	0.94 (0.38, 2.30), p=0.90	1.27 (0.78, 2.08), p=0.32	1.05 (0.94, 1.16), p=0.34	1.44 (0.92, 2.25), p=0.10
6-month mortality	**HR (95% CI)**	1.17 (0.27, 5.01), p=0.82	1.50 (0.64, 3.52), p=0.34	**1.32 (1.10, 1.59), p=0.002**	1.86 (0.82, 4.19), p=0.13
	aHR (95% CI)	1.04 (0.24, 4.44), p=0.96	0.94 (0.38, 2.32), p=0.90	**1.27 (1.07, 1.50), p=0.006**	1.78 (0.79, 4.01), p=0.16
Mean days hospitalized	**Coeff (95%CI)**	1.67 (-9.28, 12.63), p=0.76	3.43 (-2.75, 9.61), p=0.27	-0.73 (-2.14, 0.67), p=0.30	1.87 (-3.47, 7.23), p=0.48
	aCoeff (95%CI)	1.50 (-9.59, 12.59), p=0.78	3.70 (-2.87, 10.28), p=0.26	-0.73 (-2.18, 0.71), p=0.31	1.89 (-3.51, 7.30), p=0.48

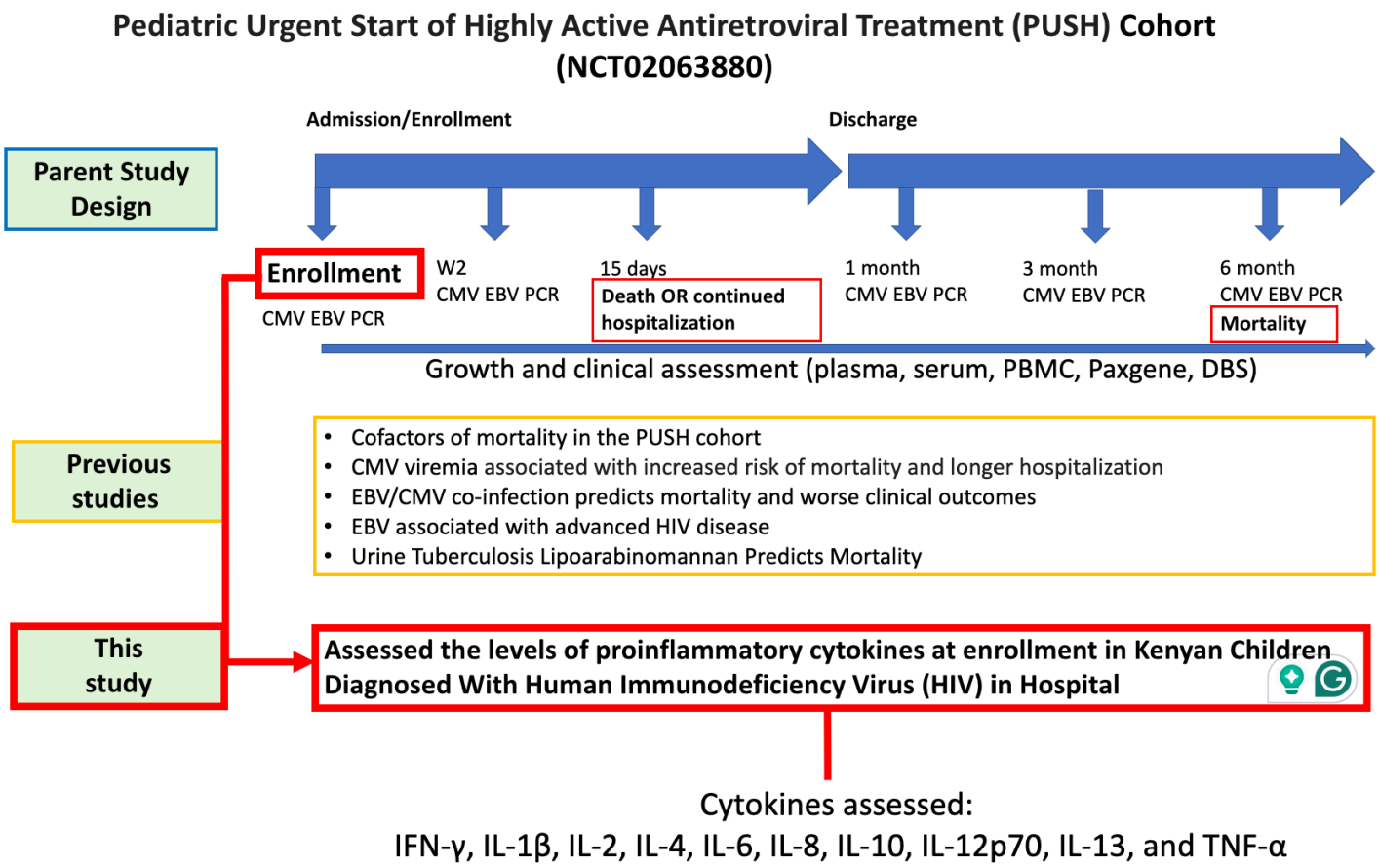
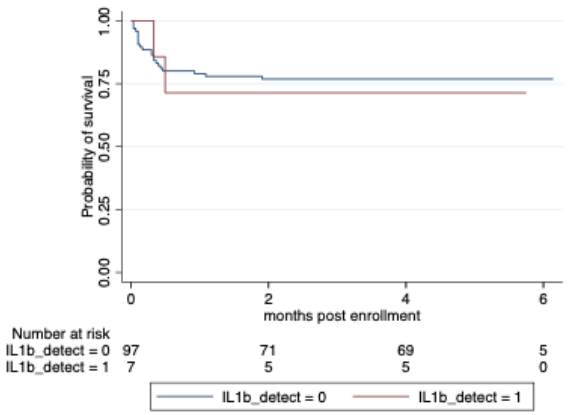
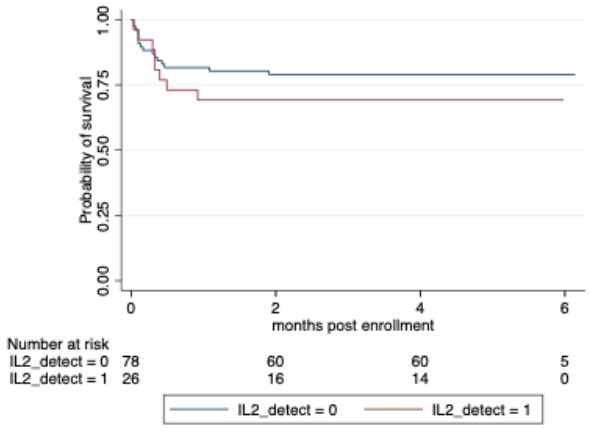


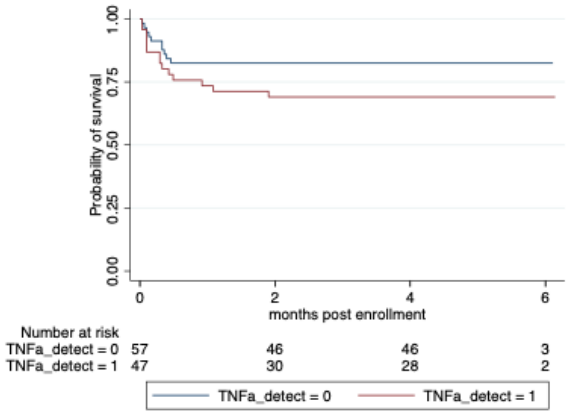
Figure 1: Schematic of the PUSH study design, follow-up schedule, and assessments.



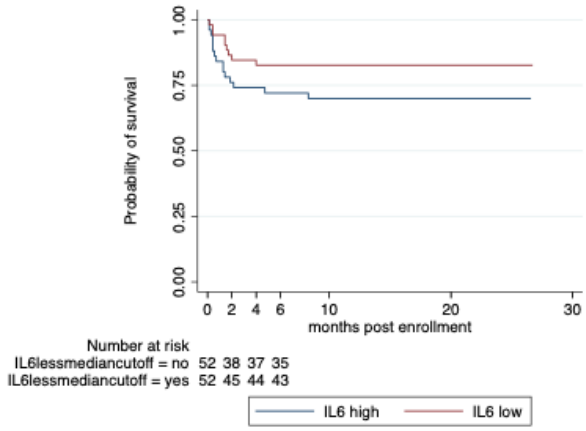
A) Log-rank test (Pr>chi2) = 0.82



B) Log-rank test (Pr>chi2) = 0.33



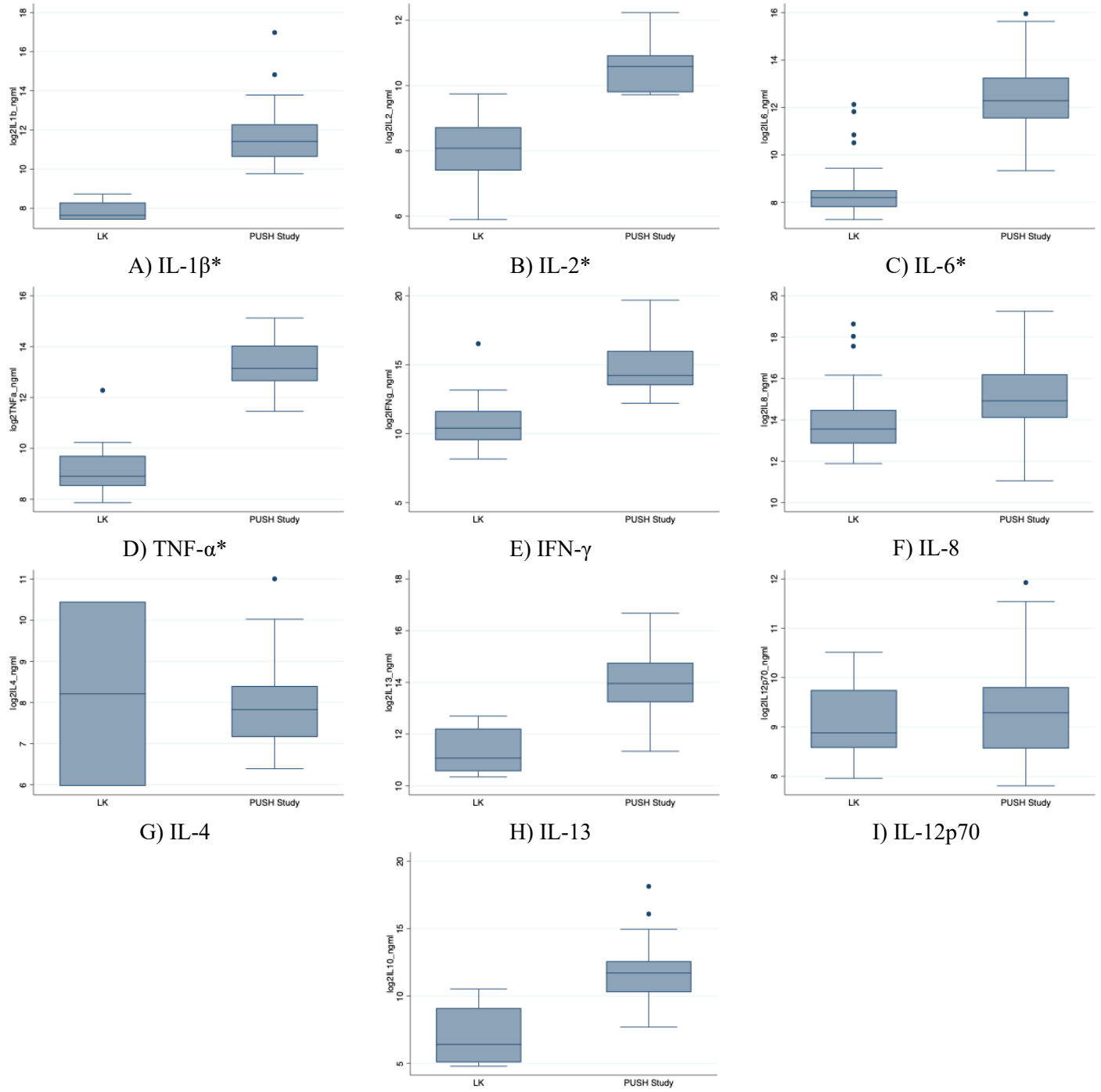
C) Log-rank test (Pr>chi2) = 0.12



D) Log-rank test (Pr>chi2) = 0.12

Figure 2: Kaplan-Meier survival probabilities for hospitalized children by admission cytokine detection. Graphs show data for primary cytokines. A) IL-1β, 24 mortalities occurred, 2 in IL-1β detected and 22 in IL-1β not detected. B) IL-2, 24 mortalities occurred, 8 in IL-2 detected and 16 in IL-2 not detected. C) TNF-α, 24 mortalities occurred, 14 in TNF-α detected and 10 in TNF-α not detected, D) IL-6, 24 mortalities occurred, 9 in IL-2 high or above median and 15 in IL-6 below median.

Supplementary Data:



A) IL-1β* B) IL-2* C) IL-6*

D) TNF-α* E) IFN-γ F) IL-8

G) IL-4 H) IL-13 I) IL-12p70

J) IL-10

Supplementary Figure 1: Plasma cytokine levels measured in 36 ART-naïve CLHIV enrolled in PUSH Study compared to 36 healthy HIV-uninfected children under 2 years old enrolled from a community clinic, in Nairobi, Kenya from Linda Kizazi (LK) study. Graph shows cytokine levels among children with detectable levels only. *Primary cytokines

Supplementary Table 2: Descriptive summary of cytokine detection and levels in serum of ART-naïve Kenyan children at hospital admission, secondary analysis.

Cytokine	Detectable n (%)	log_2 ng/mL (among detectable) Median IQR (25%, 75%)
IFN-γ	57 (54.81)	11.88 (11.36, 13.50)
IL-4	58 (55.77)	6.66 (5.65, 7.73)
IL-8	103 (99.04)	16.32 (15.14, 17.98)
IL-10	48 (46.15)	8.61 (8.09, 9.69)
IL-12p70	74 (71.15)	8.97 (8.41, 9.79)
IL-13	50 (48.08)	12.61 (12.04, 13.34)

Supplementary Table 3: Correlates of secondary cytokines among ART-naïve Kenyan children at hospital admission.

Factors	IFN-γ (detection)	IL-4 (detection)	log_2 IL-8 level	IL-10 (detection)	IL-12p70 (detection)	log_2 IL-12p70 level	IL-13 (detection)
	Adjusted PR (95% CI)	Adjusted PR (95% CI)	Adjusted Coefficient (95% CI)	Adjusted PR (95% CI)	Adjusted PR (95% CI)	Adjusted Coefficient (95% CI)	Adjusted PR (95% CI)
Age under 2	1.40 (0.73, 2.70)	0.88 (0.56, 1.37)	0.44 (-1.04, 1.94)	0.62 (0.35, 1.10)	1.17 (0.76, 1.80)	0.35 (-0.49, 1.21)	1.19 (0.62, 2.27)
Recruitment site: Kisumu	1.19 (0.83, 1.70)	1.01 (0.73, 1.40)	0.03 (-0.94, 1.00)	0.81 (0.53, 1.24)	1.01 (0.79, 1.29)	-0.20 (-0.76, 0.35)	0.81 (0.54, 1.23)
Underweight	1.12 (0.77, 1.63)	1.06 (0.75, 1.49)	0.90 (-0.12, 1.92)	0.97 (0.63, 1.49)	1.05 (0.82, 1.35)	0.14 (-0.45, 0.74)	1.14 (0.73, 1.76)
Wasted	0.93 (0.66, 1.33)	0.80 (0.56, 1.16)	0.39 (-0.81, 1.60)	0.83 (0.50, 1.37)	1.10 (0.86, 1.42)	0.34 (-0.36, 1.05)	1.10 (0.67, 1.82)
Stunted	0.96 (0.68, 1.35)	0.94 (0.66, 1.32)	0.60 (-0.39, 1.59)	1.31 (0.81, 2.13)	1.03 (0.80, 1.34)	0.24 (-0.32, 0.81)	1.25 (0.79, 1.97)
SAM	0.82 (0.52, 1.28)	0.74 (0.47, 1.17)	0.67 (-0.66, 2.01)	0.94 (0.55, 1.60)	1.10 (0.86, 1.41)	0.22 (-0.55, 1.01)	1.21 (0.73, 1.99)
WHO Stage IV	1.28 (0.83, 1.99)	1.03 (0.71, 1.51)	0.49 (-0.56, 1.56)	0.86 (0.56, 1.33)	0.96 (0.73, 1.25)	-0.07 (-0.68, 0.54)	0.93 (0.60, 1.43)
WHO Stage I/II	1.00 (0.77, 1.30)	0.82 (0.65, 1.04)	-0.06 (-0.78, 0.64)	0.91 (0.67, 1.24)	0.92 (0.77, 1.10)	-0.16 (0.57, 0.24)	0.87 (0.64, 1.18)
Severe immunosuppression	0.89 (0.57, 1.39)	0.70 (0.45, 1.09)	-0.11 (-1.34, 1.12)	0.89 (0.52, 1.52)	0.88 (0.64, 1.22)	-0.29 (-1.00, 0.40)	1.01 (0.59, 1.72)
On steroids at enrollment	1.42 (0.86, 2.36)	1.17 (0.72, 1.91)	0.21 (-1.84, 2.27)	0.70 (0.20, 2.35)	1.13 (0.84, 1.54)	0.19 (-0.96, 1.35)	1.10 (0.47, 2.60)
Total neutrophil count	0.96 (0.92, 1.00)	0.96 (0.93, 1.00)	0.04 (-0.02, 0.12)	1.01 (0.99, 1.03)	0.98 (0.95, 1.02)	-0.019(-0.061, 0.02)	0.99 (0.95, 1.02)
C-reactive protein	0.99 (0.99, 1.00)	1.00 (0.99, 1.00)	0.00 (-0.00, 0.013)	1.00 (0.99, 1.00)	0.99 (0.99, 1.00)	-0.002 (-0.009, 0.004)	0.99 (0.99, 1.00)
Hemoglobin level (g/dL)	0.96 (0.88, 1.06)	0.93 (0.84, 1.01)	-0.012 (-0.25, 0.23)	0.93 (0.83, 1.04)	1.01 (0.94, 1.08)	0.05 (-0.08, 0.19)	0.95 (0.86, 1.05)
CD4%	1.01 (0.99, 1.03)	1.00 (0.98, 1.02)	0.037 (-0.01, 0.90)	1.01 (0.99, 1.03)	1.00 (0.98, 1.01)	0.008 (-0.02, 0.03)	1.00 (0.98, 1.02)
CD4% >=15%	1.30 (0.91, 1.86)	1.08 (0.77, 1.51)	0.93 (-0.045, 1.91)	1.46 (0.95, 2.25)	1.17 (0.92, 1.49)	0.39 (-0.16, 0.96)	1.11 (0.73, 1.68)
Log10 HIV RNA copies/ml	0.98 (0.80, 1.20)	0.93 (0.77, 1.12)	-0.48 (-1.09, 0.13)	1.05 (0.80, 1.39)	1.01 (0.86, 1.20)	-0.097 (-0. I 45, 0.25)	0.93 (0.72, 1.19)
CMV >=1000 copies/ml	1.12 (0.72, 1.73)	1.10 (0.66, 1.85)	0.65 (-0.85, 2.17)	1.58 (0.94, 2.65)	1.18 (0.86, 1.62)	**0.82 (0.00, 1.64)****	1.30 (0.62, 2.73)
CMV (log10 copies/ml)	1.12 (0.86, 1.46)	1.04 (0.78, 1.38)	0.41 (-0.52, 1.35)	1.33 (0.95, 1.87)	0.98 (0.86, 1.13)	0.30 (-0.14, 0.75)	0.95 (0.67, 1.35)
EBV >=1000 copies/ml	1.10 (0.73, 1.66)	1.31 (0.83, 2.06)	0.91 (-0.23, 2.07)	1.14 (0.67, 1.92)	0.90 (0.65, 1.25)	-0.04 (-0.70, 0.60)	0.99 (0.52, 1.88)
EBV (log10 copies/ml)	1.05 (0.85, 1.31)	**1.29 (1.06, 1.56)****	0.23 (-0.48, 0.94)	0.91 (0.66, 1.25)	1.01 (0.87, 1.17)	0.15 (-0.19, 0.51)	0.92 (0.63, 1.35)

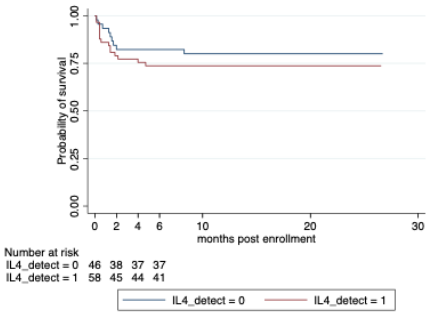
Notes. Only adjusted measures presented for readability. **p-value <0.05

Supplementary Table 4: Survival outcomes for secondary cytokines among ART-naïve Kenyan children at hospital admission.

Outcomes	Measure of association	Cytokine exposure variable					
		IFNg (detection)	IL-4 (detection)	Log_2 IL-8 level	IL-10 detection	Log_2 IL-12p70 level	IL-13 detection
Combined endpoint	IRR (95% CI)	1.08 (0.68, 1.71)	1.25 (0.78, 2.01)	1.07 (0.96, 1.18)	1.27 (0.81, 2.00)	1.10 (0.95, 1.27)	1.56 (0.98, 2.48)
	aIRR (95% CI)	0.96 (0.59, 1.52)	1.15 (0.70 1.87)	1.06 (0.96, 1.17)	1.23 (0.78, 1.94)	1.05 (0.90, 1.23)	1.58(1.00, 2.50)*
6-month mortality	HR (95% CI)	2.15 (0.89, 5.18)	1.38 (0.60, 3.16)	1.14 (0.97, 1.34)	1.46 (0.65, 3.27)	1.13 (0.87, 1.47)	1.89 (0.82, 4.33)
	aHR (95% CI)	1.64 (0.65, 4.13)	0.89 (0.36, 2.18)	1.12 (0.95, 1.31)	1.37 (0.61, 3.07)	1.01 (0.77, 1.32)	1.83 (0.79, 4.23)
Mean days hospitalized	Coeff (95%CI)	3.73 (-1.56, 9.02) P=0.17	2.86 (-2.48, 8.22) P=0.29	0.52 (-0.55, 1.60) P=0.33	2.06 (-3.26, 7.39) P=0.44	0.92 (-0.95, 2.80) P=0.33	5.16(-0.06, 10.39) P=0.05
	aCoeff (95%CI)	3.65 (-1.90, 9.21) P=0.19	3.07 (-2.61, 8.77) P=0.28	0.58 (-0.53, 1.69) P=0.30	2.02 (-3.36, 7.42) P=0.45	0.90 (-1.08, 2.89) P=0.36	5.35(0.04, 10.65) P=0.048*

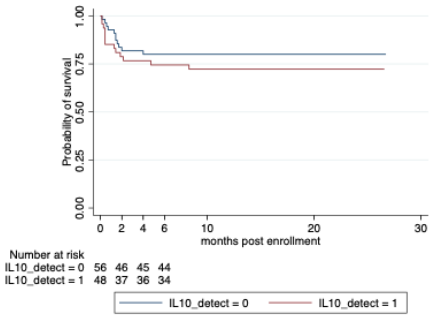
*p-value<0.05

A) IL-4



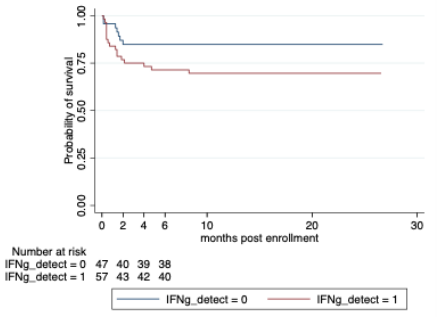
Log-rank test (Pr>chi2) = 0.44

B) IL-10



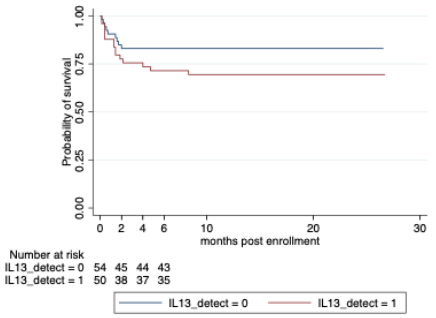
Log-rank test (Pr>chi2) = 0.34

C) IFN-g



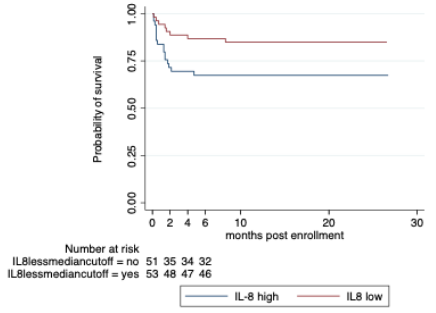
Log-rank test (Pr>chi2) = 0.08

D) IL-13



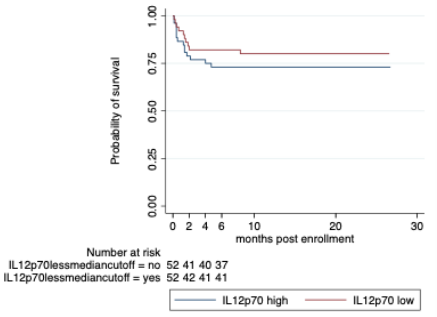
Log-rank test (Pr>chi2) = 0.12

E) IL-8



Log-rank test (Pr>chi2) = 0.03*

E) IL-12p70



Log-rank test (Pr>chi2) = 0.39

Supplementary Figure 2: Kaplan-Meier survival probabilities for hospitalized children by admission cytokine detection. Graphs show data for secondary cytokines. A) IL-4, 24 mortalities occurred, 15 in IL-4 detected *(red line)* and 9 in IL-4 not detected *(blue line).* B) IL-10, 24 mortalities occurred, 13 in IL-10 detected *(red line)* and 11 in IL-10 not detected *(blue line).* C) IFN-g, 24 mortalities occurred, 17 in IFN-g detected *(red line)* and 7 in IFN-g not detected *(blue line).* D) IL-13, 24 mortalities occurred, 15 in IL-13 detected *(red line)* and 9 in IL-13 not detected *(blue line).* E) IL-8, 24 mortalities occurred, 16 in IL-8 high or above median and 8 in IL-8 low or below median levels in ng/ml (p-value=0.03*). F) IL-12p70, 24 mortalities occurred, 14 in IL-12p70 high or above median and 10 in IL-12p70 low or below median levels in ng/ml.
*p-value<0.05

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