Non-sputum based TB diagnostics in HIV-infected children

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LIST OF ABBREVIATIONS

Ag	antigen
ART	Antiretroviral Therapy
ASU	Arizona State University
ERC	Ethics and Research Committee
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
IRB	Institutional Review Board
KNH	Kenyatta National Hospital
LAM	lipoarabinomannan
MS	Mass spectrometry
Mtb	Mycobacterium tuberculosis
PMTCT	Prevention of mother-to-child transmission
PUSH	Pediatric Urgent Start of HAART (PUSH) Study
TB	Tuberculosis
UoN	University of Nairobi
UW	University of Washington
WHO	World Health Organization
Xpert	GeneXpert MTB/RIF (TB diagnostic)

EXECUTIVE SUMMARY

Title: Non-sputum based TB diagnostics in HIV-infected children

Objective: Over one million new cases of tuberculosis (TB) and 239,000 TB-related deaths occur in children each year. Young children, especially those with HIV, are more likely to present with disseminated or extrapulmonary TB and paucibacillary disease, often missed by respiratory sampling. We propose to investigate the performance of blood-based TB diagnostics in HIV-infected children using archived specimens and data from the Pediatric Urgent Start of HAART (PUSH) Study (NCT02063880).

Our collaborators have developed blood-based methods to rapidly quantify M. tuberculosis (Mtb)-specific antigen (Ag) peptide fragments (CFP-10/ESAT-6) using antibody-labeled and energy-focusing porous discoidal silicon nanoparticles (nanodisks) with high-throughput mass spectrometry (MS) enhancing sensitivity and specificity for TB diagnosis (NanoDisk-MS). In pilot studies, NanoDisk-MS was able to detect Mtb-Ag in the blood of HIV-infected adults with sputum culture-confirmed TB, as well as those with culture-negative and extrapulmonary TB.

NanoDisk-MS features make it optimal particularly for HIV-infected children including easily collectable small blood volume requirement (<1ml) and potential for detection of paucibacillary and disseminated TB.

We propose to evaluate the performance of NanoDisk-MS detected Mtb-Ag for diagnosis (Aim 1), mortality prognosis (Aim 2), and treatment response (Aim 3) in HIV-infected children using archived specimens and data from the Pediatric Urgent Start of HAART (PUSH) Study (NCT02063880). In addition to assessing conventional diagnostic performance measures, we propose to use advanced epidemiologic methods (Bayesian latent class analysis) given the context of an imperfect reference (Exploratory aim).

Using cryopreserved samples from a well-characterized cohort of HIV-infected children who underwent intensive TB evaluation provides an opportunity for efficient evaluation of a novel diagnostic with potential for clinical impact to improve TB diagnosis in HIV-infected children globally.

Overall objective: The study aims to evaluate the performance of a novel blood-based TB diagnostic on cryopreserved samples from a

well-characterized cohort of HIV-infected children who underwent intensive TB evaluation.

Aims:	<u>Specific aims</u>: **Aim 1. Determine diagnostic performance of NanoDisk-MS to identify active TB in HIV-infected children.** **Aim 2. Determine prognostic performance of NanoDisk-MS to identify HIV-infected children at greatest risk of death.** **Aim 3. Determine NanoDisk-MS utility to assess TB treatment response in HIV-infected children.**
Methods	Secondary analysis of existing specimens from completed studies will include experiments using blood samples. The proposed studies will use stored specimens from the completed the Pediatric Urgent Start of HAART (PUSH) Study (NCT02063880) conducted by the UW-University of Nairobi collaborative group. **PUSH Study:** UW#STUDY00001052, "Post-Stabilization vs Urgent Start of HAART in HIV-1 Infected Children with Severe Co-infections" <u>*Lead researchers*</u>: Grace John-Stewartand (UW), Dalton Wamalwa (UoN)
Population:	HIV-infected hospitalized children
Sites:	Kenyatta National Hospital, Kisumu District Hospital, Nyanza Provincial Hospital, Mbagathi District Hospital
Study Duration:	This is a 2 year project. Children in the cohorts to be studied were followed up for 24 weeks after enrollment.
Outcomes	**Aim 1. Determine diagnostic performance of NanoDisk-MS to identify active TB in HIV-infected children.** <u>*Hypothesis*</u>: NanoDisk-MS will have similar performance to culture/Xpert among children with confirmed TB without need for sputum. <u>*Approach:*</u> We will compare sensitivity, specificity, negative and positive predictive values, and AUC of NanoDisk-MS to reference of culture/Xpert. Exploratory: <u>*Hypothesis:*</u> NanoDisk-MS will identify additional children with TB missed by conventional respiratory tests, especially younger children and those with severe immunosuppression. <u>*Approach*</u>: We will assess NanoDisk-MS diagnostic performance using Bayesian latent class analysis, an analytic approach that can be used in the setting of imperfect reference tests. **Aim 2. Determine prognostic performance of NanoDisk-MS to identify HIV-infected children at greatest risk of death.** <u>*Hypothesis:*</u> NanoDisk-MS will identify children who died, including those missed by conventional TB diagnostics on respiratory samples.

Approach: We will compare overall 6-month mortality among children by enrollment NanoDisk-MS result.

Aim 3. Determine NanoDisk-MS utility to assess TB treatment response in HIV-infected children. *Hypothesis:* Mtb-Ag as measured by NanoDisk-MS will decline in children with successful TB treatment outcomes. *Approach:* We will analyze Mtb-Ag levels longitudinally among children initiating TB treatment and compare between children with and without clinical improvement.

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BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

A. SUMMARY

Of the 239,000 yearly TB-associated pediatric deaths, 80% are <5 years of age, and 96% die without initiating treatment.[1,2] Young children, especially with HIV, are more likely to present with disseminated or extrapulmonary TB and paucibacillary disease, potentially missed by respiratory sampling.[3,4] Culture, the gold standard, takes weeks to result and is positive in only 30-60% of pediatric TB.[5,6] WHO recommended TB diagnostic Xpert MTB/RIF reduces time to result, but has decreased sensitivity in children (62-66%), is associated with sampling challenges, and can remain positive after treatment decreasing utility for treatment response monitoring.[7-9]

In the completed Pediatric Urgent vs. post-Stabilization HAART initiation (PUSH) trial, hospitalized HIV-infected children underwent intensive TB investigations.[10] Six-month mortality (61/100 PY) and prevalence of confirmed TB (8%) by Xpert/culture were high. Importantly, Xpert performed as well on stool as respiratory samples, however 44% of children with signs/symptoms suggestive of TB still had 'unconfirmed TB' with negative Xpert/culture.[11] Urine lipoarabinomannan (LAM) had modest diagnostic performance, but strongly predicted mortality (aHR 4.9 for LAM+ vs. LAM-).[12] Notably, risk of death was especially high (>10-fold) for LAM+ children with unconfirmed TB missed by respiratory samples. **Diagnostic tools for rapid TB detection and treatment response in children, using easily obtained specimens are urgently needed, especially those which identify children at highest risk of death.**

Our collaborators (Hu, Co-I) have developed a blood-based method to rapidly quantify *M. tuberculosis* (Mtb)-specific antigen (Ag) peptide fragments (CFP-10/ESAT-6) using antibody-labeled and energy-focusing porous discoidal silicon nanoparticles (nanodisks) with high-throughput mass spectrometry (MS).[13-16] NanoDisk-MS diagnosed pulmonary TB with high sensitivity in culture-confirmed HIV- (93%) and HIV+ (91%) adults (specificity 87-100%), and detected Mtb-Ag in blood of extrapulmonary and culture-negative TB cases. Intriguingly, higher Mtb-Ag levels in HIV-infected adults suggest enhanced performance in this group, and decreased Mtb-Ag during TB therapy was associated with successful treatment outcomes. This may be of particular benefit to children, in whom sputum confirmation is typically lacking at diagnosis and during treatment.

NanoDisk-MS features may make it exceptionally well suited for HIV-infected children including small blood volume requirement (<1ml) and improved performance in paucibacillary and disseminated TB. The extensively characterized PUSH cohort with longitudinally collected cryopreserved samples provides opportunity for **efficient evaluation of NanoDisk-MS for TB diagnosis and treatment response in young, immunosuppressed children with varied clinical presentations at high risk for death**

We propose to evaluate the performance of NanoDisk-MS detected Mtb-Ag for diagnosis (Aim 1), mortality prognosis (Aim 2), and treatment response (Aim 3) in HIV-infected children using archived specimens and data from the Pediatric Urgent Start of HAART (PUSH) Study (NCT02063880). In addition to assessing conventional diagnostic performance measures, we propose to use advanced epidemiologic methods (Bayesian latent class analysis) given the context of an imperfect reference (Exploratory aim).

Using cryopreserved samples from a well-characterized cohort of HIV-infected children who underwent intensive TB evaluation provides an opportunity for efficient evaluation of a novel diagnostic with potential for clinical impact to improve TB diagnosis in HIV-infected children globally.

Overall objective: The study aims to evaluate the performance of a novel blood-based TB diagnostic on cryopreserved samples from a well-characterized cohort of HIV-infected children who underwent intensive TB evaluation.

B. INTRODUCTION

Tuberculosis (TB) contributes to significant morbidity and mortality in children globally.[1,17] The World Health Organization (WHO) estimates one million new TB cases in children each year.[2] Recent models suggest the majority of 239,000 yearly pediatric TB deaths are in children <5 years (80%), untreated for TB (96%) (**Fig. 1**).[1] Pediatric TB-related mortality is likely underreported due to detection difficulties, and is among top causes of respiratory death in sub-Saharan Africa necropsy studies.[18,19] **Identifying children with TB is key to initiating life-saving therapy and reducing mortality.**
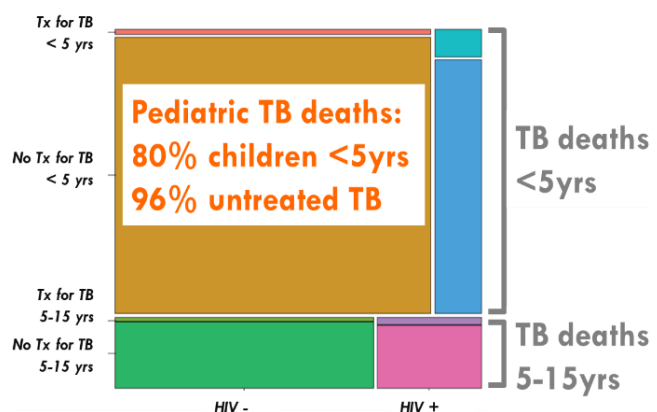

Fig. 1: The majority of pediatric TB deaths occur in children under 5 years, who are untreated for TB. Adapted from **Dodd Lancet Global Health 2017**

Sputum-based diagnostics underestimate TB in HIV-infected children. Young children, especially those with HIV, often have paucibacillary disease and are more likely to present with disseminated or extrapulmonary TB, difficult to identify by respiratory sampling.[4,5,20] Challenges in obtaining respiratory samples and rapid disease progression lead to treatment delays and poor outcomes.[21] Culture, the gold standard for pulmonary TB diagnosis, is positive in only 30-62% of pediatric cases.[6] Despite recent advances in rapid TB diagnostics such as Xpert,[7] sensitivity is reduced in children (62-66% vs. culture).[7,9] WHO-recommended Xpert samples include sputum which often cannot be feasibly collected in young children, gastric aspirates which require fasting and frequently hospitalization, and induced sputum which can result in adverse events and potential transmission risk.[22,23] Furthermore, for young children in whom primary TB is often restricted to hilar lymph nodes,[5,24] in whom bacilli may be absent or minimal in respiratory secretions, there is need to explore alternative specimens. **Non-sputum based diagnostic tools, including those for treatment monitoring are urgently needed for children.**[3,25]

Currently available non-sputum based diagnostics reduce sampling challenges, but still miss pediatric TB cases. We and others have reported performance of Xpert on stool and LAM in urine for pediatric TB (**Table. 1**).[7,9,22,26-35] LAM likely detects Mtb in urine after dissemination accounting for its increased performance in HIV-infected adults with low CD4.[36] Conversely, stool Xpert likely only identifies children with detectable TB in sputum that has been swallowed then transported through the gastrointestinal tract, thus missing paucibacillary,

Diagnostic	Sputum/ GA Cx	Sputum/ GA Xpert	Stool Xpert	Urine LAM	NanoDisk-MS Blood
Collection ease	−	−	+	+	+
Low processing requirement	−	+	−	+	−***
Able to detect dissem. TB	−	−	−	+	+
Treatment response monitoring	+*	−	−	−	+
Performance compared to culture	Reference**	Sens 51-81% Spec 96-99%	Sens 31-90% Spec 98-100%	Sens 43-100% Spec 73-90% *HIV+ only*	*Unknown in children*

GA: gastric aspirate   * in practice rarely done ** thought to miss 30-62% of peds TB cases
***POC development efforts ongoing with Nanopore platform

Table 1. *Characteristics and performance of selected TB diagnostics in children*

extrapulmonary, or disseminated disease. Although stool is easily obtainable from young children, sample processing remains intensive and time consuming.[26] Urine LAM dipstick is simple to use, and can identify children with disseminated disease and those at high risk of mortality,[37] but due to low sensitivity is recommended for use only in HIV-infected individuals with severe immunosuppression.[34] Neither diagnostic can be used to reliably track treatment response. Host-derived transcriptional signatures are promising[38-41] but are likely years from practical clinical use due to need for highly specialized equipment. In very young, or immunosuppressed children occult dissemination can occur early during primary Mtb infection

or disseminate hematogenously to extrapulmonary sites,[5] providing biologic plausibility for antigen detection in blood samples, despite reported low prevalence of pediatric Mtb bacteremia.[42]

M. tuberculosis (Mtb)-specific antigen peptide fragments are readily detectable in blood and can be used to diagnose TB in adults. Our collaborators (Hu) have identified Mtb-specific antigen (Ag) peptide fragments (CFP-10/ESAT-6) in blood and developed methods to rapidly quantify their concentrations, using antibody-labeled and energy-focusing porous discoidal silicon nanoparticles (nanodisks) and high-throughput mass spectrometry (MS) to enhance sensitivity and specificity.[13-16] NanoDisk-MS diagnosed active TB with high sensitivity in culture-confirmed HIV-negative (93%) and HIV-positive (91%) adults with high specificity (87-100%) in both healthy and high-risk adults.[13] **Intriguingly, significantly higher levels of combined CFP-10/ESAT-6 in this population suggest enhanced performance in HIV-infected individuals,** a group often difficult to diagnose through sputum due to paucibacillary disease. Additionally, decreased Mtb-Ag concentrations during TB therapy were associated with successful treatment outcomes, which may be of particular benefit to children, in whom sputum diagnostic confirmation is typically lacking at presentation and during treatment. Diagnostic gaps NanoDisk-MS Mtb-Ag detection addresses are ease of sample collection, potential superior performance, and treatment response monitoring.

Pediatric TB diagnostics We evaluated TB diagnostics in HIV-infected children enrolled in an ART initiation trial in Kenya (PUSH cohort).[10] Among 181 children, 165 had sputum/gastric aspirates (GA) for reference culture. Fourteen (8%) had confirmed TB (by culture and/or Xpert). Importantly, stool Xpert performed as well as sputum/GA Xpert (**Fig. 2**).[24]
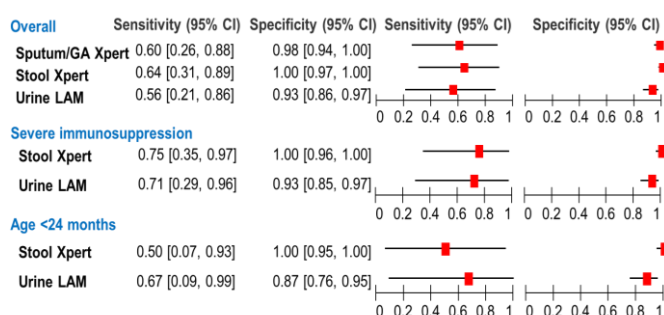
*Fig. 2. Performance of sputum/GA, stool Xpert, and urine LAM to detect TB in HIV-infected hospitalized children in Kenya. GA: gastric aspirate. **LaCourse AIDS 2018***
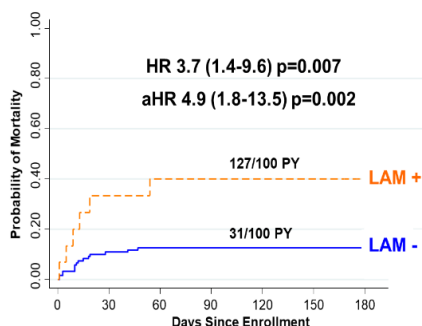
Fig. 3**. HIV-infected TB-LAM+ children had >4.9-fold risk of mortality even after adjusting for age, CD4%, and parent trial arm. **LaCourse CID 2018

Urine LAM had lower sensitivity/specificity in general, but improved in children with severe immunosuppression and younger age, likely due to hematogenous presence of Mtb due to poor containment. Despite modest diagnostic performance, urine LAM predicted overall mortality (aHR 4.9 for LAM+) (**Fig. 3**).[66] Notably, risk of death was especially high (>10-fold) for LAM+ children with unconfirmed TB. LAM-positivity was also predictive of mortality in important subgroups including those with severe HIV-immunosuppression (HR 4.7) or malnutrition (HR 5.4). These data indicate **need for a rapid, biomarker-based diagnostic using an easily obtainable sample such as blood for TB diagnosis and treatment response.**

NanoDisk-MS Mtb-Ag detection for TB diagnosis and treatment response Initial NanoDisk-MS diagnostic clinical evaluation was performed using data and samples from clinically suspected and/or laboratory-diagnosed Houston TB Initiative adult cases.[14,43] NanoDisk-MS detected 93% (25/27) of HIV-negative culture-positive cases, with 100% sensitivity in smear-positive and 91% sensitivity in smear-negative pulmonary TB cases (**Fig. 4**).[11]
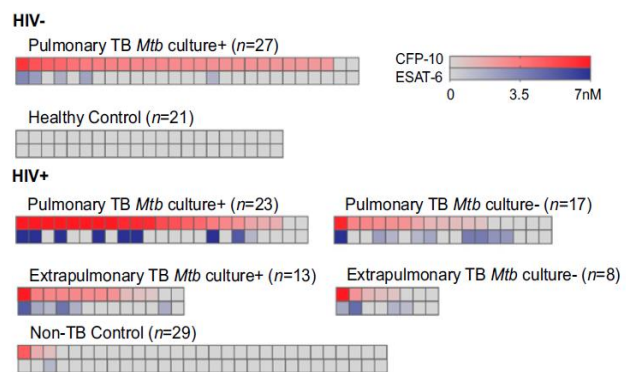
Fig. 4. NanoDisk-MS identification of active TB in adult HIV-uninfected and HIV-infected adults. Each column represents a subject with CFP-10 in red, and ESAT-6 concentration in blue. Mtb-Ag levels are indicated by color intensity. **Liu PNAS 2017**
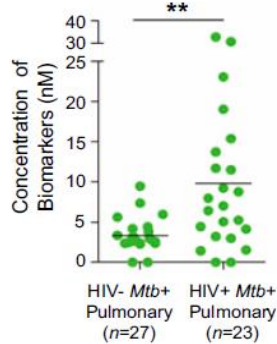
Among HIV+ participants, NanoDisk-MS identified 91% (21/23) Mtb-culture positive pulmonary and 92% (12/13) extra-pulmonary cases. NanoDisk-MS also detected Mtb-Ag in 82% (14/17) culture- negative pulmonary and 75% (6/8) extrapulmonary TB cases, suggesting NanoDisk-MS detects patients missed by culture. Specificity was 100% in HIV- and 90% in HIV+ controls. Notably, combined Mtb-Ag concentrations (CFP-10/ESAT-6) were higher among HIV-infected vs. uninfected culture-confirmed TB patients (**Fig. 5**), indicating performance may be enhanced in this group often difficult to diagnose due to paucibacillary disease.

Fig. 5: NanoDisk-MS measured Mtb-Ag (CFP-10/ESAT-6) in HIV+ and HIV- culture-confirmed adult TB cases.

In further evaluation, combined Mtb-Ag responses did not differ between culture-confirmed pulmonary and extrapulmonary cases and were similar in culture-negative pulmonary and extrapulmonary cases (**Fig. 6**), suggesting Mtb-Ag is detectable in peripheral blood across a wide range of clinical presentations, including those typically missed by respiratory sampling.[13]
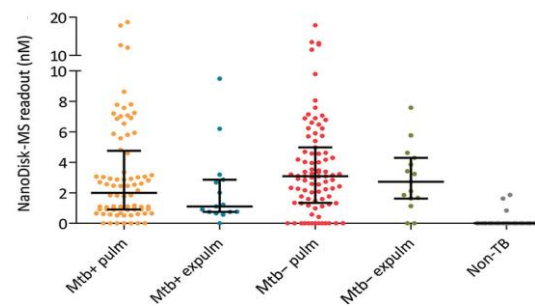
Fig. 6. CFP-10/ESAT-6 Ag are detectable by NanoDisk-MS and similar for culture-confirmed pulmonary, extrapulmonary, and culture-negative adult TB cases.

NanoDisk-MS Mtb-Ag concentrations decreased with TB treatment in HIV- TB cases, suggesting utility to monitor treatment response (**Fig. 7**).[11] **NanoDisk-MS provides a sensitive and specific means of rapid Mtb-Ag detection using small volumes of readily available sample type (blood) in HIV-positive individuals, with potential for treatment monitoring.**
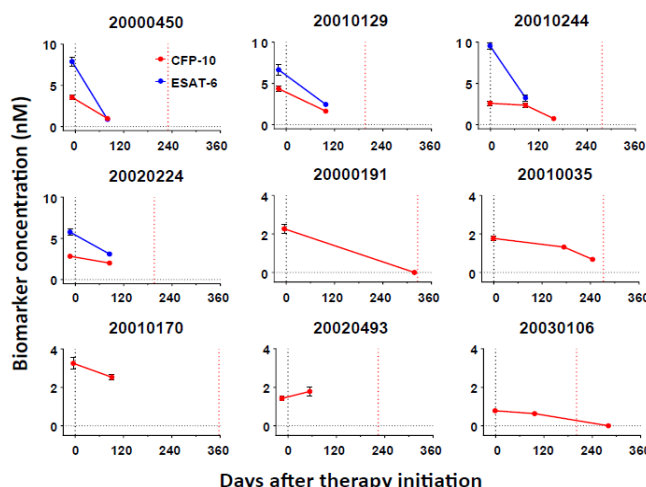
Fig. 7. Serial NanoDisk-MS quantification of CFP-10 and ESAT-6 for TB treatment response evaluation in adult HIV- TB cases.

Clinical cohort

The Pediatric Urgent Start of HAART (PUSH) Study
(PI John-Stewart, [Co-I] Wamalwa) ascertained whether urgent (<48 hours) vs. post-stabilization ART (7-14 days) improved survival in hospitalized children <12 years old. In this young, malnourished cohort with severe immunosuppression, overall mortality was 22% (**Table 2**). Urgent ART neither improved nor decreased survival.[10] Confirmed (8%) and unconfirmed TB (43%) prevalence was high. Children underwent clinical examination on enrollment, 1, 2, 4, 12, and 24 weeks. Plasma and serum were serially collected and cryopreserved (**Table 4** in Methods).

TB investigations and treatment On enrollment children were systematically screened for TB symptoms and exposure, and sputum or gastric aspirates for Xpert and culture, urine for LAM, and stool for Xpert collected irrespective of TB symptoms.[26] CXR were read by a radiologist using standardized forms.[44] Children were categorized as confirmed, unconfirmed, or unlikely TB based on international consensus.[43] Diagnostic results were available to study clinicians; TB treatment was initiated per Kenyan guidelines.[45]

Table 2. PUSH participant characteristics	
N=181	**n or median (% or IQR)**
Age (years)	1.9 (0.8-4.8)
Female	81 (45)
Malnourished (WAZ <-2)	113 (65)
CD4 %	14.5 (9.0-22.0)
Severe immunosuppression	125 (69)
TST+ (n=160)	
TB exposure (n=178)	22 (12)
CXR suggestive TB (n=154)	91 (59)
TB treatment (n=177)	63 (36)
Died	39 (22)
TB clinical case definition[43]	
Confirmed	14 (8)
Unconfirmed	78 (43)
Unlikely	89 (49)
WAZ: weight for age Z score	

RATIONALE TB causes significant morbidity and mortality in children, particularly those with HIV often missed by respiratory sampling. Using cryopreserved samples from a well-characterized cohort of hospitalized children who underwent intensive TB evaluation provides opportunity for efficient evaluation of diagnostic approach with likelihood of high clinical impact for one of the leading causes of infectious mortality in HIV-infected children.

Anticipated outcomes, potential problems, and alternative approaches. We anticipate NanoDisk-MS will 1) have similar performance to Xpert/culture in children with confirmed TB without need for sputum, 2) identify additional children missed by the reference including those at highest risk of death, and 3) provide a useful surrogate marker of treatment response. We are well-powered to meet our Aim 1 and 2 objectives based on pilot NanoDisk-MS adult data and previous pediatric PUSH cohort diagnostic work. Aim 3 may be more limited by longitudinal 12 and 24 week sample availability, however we will not limit our analysis to culture-confirmed TB given culture misses 30-60% of pediatric TB. Samples available at other time points may reveal interesting Mtb-Ag response patterns regardless. Current consensus criteria recommend pediatric TB treatment response can be appropriately evaluated at 2 months after treatment initiation with additional suggested evaluation at 6 months, as we propose in Aim 3.[67] Exploratory LCA estimate precision will likely be higher with inclusion of additional clinical characteristics and diagnostic tests in our models. We may gain power by assessing NanoDisk-MS results as continuous as opposed to dichotomous positive/negative results. Identification of drug resistance mutations is important (a current limitation of NanoDisk-MS), however the majority of new TB cases and TB-related deaths in children are due to drug susceptible TB that remains undiagnosed with current diagnostics and therefore untreated.[1,2,97] An additional benefit of NanoDisk-MS for treatment response could be to facilitate regimen switch as most children respond favorably to MDR-TB treatment even without MDR microbiologic confirmation.[97,98] Alternative approaches to peptide capture

(nanopore sensing) are under evaluation allowing for future efficient assessment of our clinical specimens while utilizing a similar rigorous analysis approach.

C. OBJECTIVES

1.1 General Objective
The study aims to evaluate the performance of a novel blood-based TB diagnostic using soted de-ientified samples from a completed well-characterized cohort of HIV-infected children who underwent intensive TB evaluation.

1.2 Specific Aims

Aim 1. Determine diagnostic performance of NanoDisk-MS to identify active TB in HIV-infected children. *Hypothesis:* NanoDisk-MS will have similar performance to culture/Xpert among children with confirmed TB without need for sputum. *Approach:* We will compare sensitivity, specificity, negative and positive predictive values, and AUC of NanoDisk-MS to reference of culture/Xpert. Exploratory: *Hypothesis:* NanoDisk-MS will identify additional children with TB missed by conventional respiratory tests, especially younger children and those with severe immunosuppression. *Approach:* We will assess NanoDisk-MS diagnostic performance using Bayesian latent class analysis, an analytic approach that can be used in the setting of imperfect reference tests.

Aim 2. Determine prognostic performance of NanoDisk-MS to identify HIV-infected children at greatest risk of death. *Hypothesis:* NanoDisk-MS will identify children who died, including those missed by conventional TB diagnostics on respiratory samples. *Approach:* We will compare overall 6-month mortality among children by enrollment NanoDisk-MS result.

Aim 3. Determine NanoDisk-MS utility to assess TB treatment response in HIV-infected children. *Hypothesis:* Mtb-Ag as measured by NanoDisk-MS will decline in children with successful TB treatment outcomes. *Approach:* We will analyze Mtb-Ag levels longitudinally among children initiating TB treatment and compare between children with and without clinical improvement.

D. METHODS

1.3 Study Design
Our overall goal is to evaluate to evaluate the performance of a novel blood-based TB diagnostic using cryopreserved samples from a well-characterized cohort of HIV-infected children who underwent intensive TB evaluation (**Table 3**). The

Table 3. Overall Study Strategy	
Study Design	TB diagnostic study utilizing clinical data and respository samples from a completed pediatric ART initiation trial in Kenya
Aims	AIM 1: Diagnostic performance of NanoDisk-MS to detect TB AIM 2: Prognostic performance of NanoDisk-MS to predict mortality AIM 3: Utility of NanoDisk-MS for TB treatment response
Population	HIV-infected hospitalized children $\leq$ 12 years of age
Exclusions	Children with CNS infection (parent trial exclusion)
Follow-up	6 months post-enrollment
Sampling framework	All children enrolled in parent trial with cryopreserved samples

studies described here are all secondary analyses of existing de-identified stored specimens from completed studies. **There will be no additional sample or clinical data collection for these studies.**

1.4   Site

All studies will be retrospective on stored existing specimens stored at the University of Nairobi or at the University of Washington/Fred Hutchinson Cancer Research Center in Seattle. NanoDisk-MS assays will be performed in the laboratory of Dr. Ye Hu at Arizona State University.

1.5   Population

The proposed studies will use stored blood specimens from the completed the Pediatric Urgent Start of HAART (PUSH) Study (NCT02063880) conducted by the UW-University of Nairobi collaborative group. All data and samples used for this study are deidentified.

PUSH Study: UW#STUDY00001052 UoN/KNH ERC P378/09/2011, "Post-Stabilization vs Urgent Start of HAART in HIV-1 Infected Children with Severe Co-infections" *Lead researchers*: Grace John-Stewartand (UW), Dalton Wamalwa (UoN)

1.5.1   Inclusion Criteria

o   Previously enrolled in the PUSH study described above.

1.5.2   Exclusion Criteria

o   Participants with no specimen available.

1.5.3   Recruitment and Retention

Not applicable. All studies will be on archived de-identified samples previously collected.

1.6   Sample Size and Framework

Our plan is to select samples from participants of completed studies including:

PUSH cohort: 181 children (Table 4)

Table 4. Timepoints of available samples for Aims					
Timing (weeks)	**0**	**2**	**4**	**12**	**24**
Total samples*	179	139	133	132	115
Aim 1					
Confirmed TB	14	10	10	6	5
Unconfirmed TB	74	62	60	62	53
Unlikely TB	91	67	62	64	57
Aim 2					
Survived	140	N/A			
Died	39				
Aim 3					
TB treatment	60	54	54	52	44
No TB treatment	108	82	75	77	70
*Serum and/or plasma					
Proposed study will utilize samples in grey					

1.7   Data analysis

Aim 1. Determine diagnostic performance of NanoDisk-MS to identify TB in HIV-infected children. NanoDisk-MS diagnostic performance will be compared to reference of Xpert/culture. Mtb detection in >1 sample by Xpert/culture will be considered confirmed TB. Any NanoDisk-MS detectable CFP-10/ESAT-6 will be considered positive. Since the reference misses true cases of pediatric TB, we will perform sensitivity analyses using a composite reference standard[46] of Xpert/culture or NanoDisk-MS positive.

Primary analyses: Sensitivity, specificity, positive and negative predictive values, and AUC of NanoDisk-MS vs. reference Xpert/culture to diagnose TB will be estimated using 95% confidence intervals (CI) assuming binomial distribution.

Sample size and power: Estimated precision of NanoDisk-MS performance for a range of TB

Table 5. Estimated precision of NanoDisk-MS (ND-MS) performance for different prevalence, sensitivity and specificity estimates (n=179)			
TB Prev %	Est. ND-MS Positive Cases (%)	Est. ND-MS Sensitivity % (95% CI)	Est. ND-MS Specificity % (95% CI)
AIM 1: Xpert/culture reference			
8*	13 (7.6)	95 (66.1-99.8)	98 (94.8-99.6)
8	13 (7.2)	90 (66.1-99.8)	95 (90.7-97.9)
8	12 (6.8)	85 (57.2-98.2)	90 (84.7-94.4)
AIM 1 Exploratory: Composite Xpert/culture & NanoDisk-MS reference			
10**	16 (8.9)	90 (65.3-98.6)	98 (94.7-99.6)
15	24 (13.4)	90 (70.8-97.6)	95 (89.9-97.7)
20	32 (17.9)	90 (73.9-96.9)	90 (84.1-94.5)
*Xpert/culture positive			
**Xpert/culture + est. additional NanoDisk-MS positive			
Most likely estimates in grey			

prevalence and sensitivity/specificity estimates are described in **Table 5**. Given 8% confirmed TB prevalence by Xpert/culture and assuming NanoDisk-MS 90% sensitivity based on performance in HIV-infected adults, 95%CI would be 66.1-99.8% for sensitivity, and 90.7-97.9% for 95% specificity. Additional estimates are provided for exploratory analyses assuming increased case detection with composite reference of Xpert/culture and NanoDisk-MS.

Sensitivity analyses: We will repeat above analyses using a composite Xpert/culture and NanoDisk-MS reference standard.

Exploratory analyses: To explore possible differences in performance in populations of children with high risk of disseminated TB, we will stratify by HIV immunosuppression (severe vs. not severe[47]), and age (<24 vs. >24 months).

Latent Class Analysis: Pediatric TB diagnosis currently relies on combination of imperfect tests likely underestimating true prevalence. To address this, we will use Bayesian latent class analysis (LCA), an approach used in the setting of an imperfect reference standard,[48] including pediatric TB[49,50] to estimate TB prevalence and diagnostic performance of NanoDisk-MS and references Xpert/culture. With this approach, Schumacher et al. estimated 60% sensitivity for culture and 49% for Xpert in 749 hospitalized South African pediatric TB suspects, suggesting these references miss 40-51% of pediatric cases.[49]

<u>Analyses:</u> We will develop a heuristic model of available clinical and diagnostic information with the goal of classifying children into latent classes of "true" TB and not TB. Using a Bayesian approach, we will estimate probability of true TB based on different assumptions of independence between test outcomes and assess sensitivity to our prior assumptions. We assume covariates of interest will influence TB prevalence and test accuracy (age, sex, HIV-immunosuppression, severe malnutrition, TB contact) and will assess model variations through covariate adjustment.

Table 6 lists average posterior means of "true" TB prevalence, diagnostic performance, and probability of 95% posterior credible intervals (CrI) including parameter values listed ("coverage") from 1000 simulated datasets based on 17% true TB prevalence (assuming culture identified 60% and Xpert 40% true TB), informative priors for reference Xpert/culture[7,9,49] and non-informative priors for NanoDisk-MS. While CrI coverage is below the nominal 95% level, we may still capture improved NanoDisk-MS performance relative to reference tests even under non-informative priors for NanoDisk-MS.

Table 6. Est. mean 95% CrI coverage for posterior median estimates of TB prevalence, diagnostic sensitivity and specificity for true TB using 1000 simulated data sets (n=181)			
	Prevalence (95% CrI coverage)	Sens (95% CrI coverage)	Spec (95% CrI coverage)
Scenario 1 (assuming ND-MS Sens 95%/Spec 98%)			
Prevalence	18.3 (92.9)	-	-
Culture		58.6 (96.6)	99.9 (25.9)
Xpert		41.9 (96.9)	99.6 (41.1)
NanoDisk-MS		81.5 (62.5)	96.9 (99.1)
Scenario 2 (assuming ND-MS Sens 90%/Spec 95%)			
Prevalence	19.7 (87.9)	-	-
Culture		54.6 (95.5)	99.9 (30.3)
Xpert		39.9 (96.4)	99.4 (39.1)
NanoDisk-MS		77.8 (72.1)	95.2 (98.8)
Scenario 3 (assuming ND-MS Sens 85%/Spec 90%)			
Prevalence	21.5 (80.7)	-	-
Culture		51.1 (91.4)	99.9 (36.1)
Xpert		37.0 (95.6)	99.7 (29.4)
NanoDisk-MS		73.8 (75.7)	91.4 (97.6)
Assuming true TB prevalence of 17%, culture sens 60%/spec 99%, Xpert sens 40%/spec 98% based on Schumacher et al. and published reviews[7,9,49]			

Aim 2. Determine NanoDisk-MS prognostic performance to identify HIV-infected children at greatest risk of death. We hypothesize NanoDisk-MS will identify HIV-infected children most likely to die, including those missed by conventional testing.

<u>Primary analyses:</u> We will use Cox proportional hazards regression to compare 6-month mortality incidence between children with/without NanoDisk-MS detectable Mtb-Ag on enrollment.

<u>Exploratory analyses:</u> We will stratify mortality incidence by NanoDisk-MS result by: TB status (confirmed/unconfirmed vs. unlikely TB), HIV-immunosuppression, and malnutrition. Stratified analyses may have lower power, but reveal important NanoDisk-MS performance information in subgroups at risk for disseminated or extrapulmonary TB.

<u>Sample size and power:</u>
In previous work, 6-month mortality among LAM+ children was 127/100 PY (40%) vs. 31/100PY (12%) among LAM- (aHR 4.9). Given conservative estimates of NanoDisk-MS positivity of 20-25% and mortality among NanoDisk-MS negative of 12-20%, we will have 80% power to detect HR 3.4-4.9 (**Table 7**).

Table 7. Minimal detectable HR for mortality incidence by NanoDisk-MS result (n=179)		
ND-MS+ %	ND-MS– mortality %	Min detect HR
15	12	6.1
20	12	4.9
25	12	4.3
15	15	5.2
20	15	4.4
25	15	3.9
15	20	4.5
20	20	3.8
25	20	3.4
Assuming β=0.80, 2-sided α=0.05 *Most likely estimates in grey*		

Aim 3. Determine NanoDisk-MS utility to assess TB treatment response in HIV-infected children. We will analyze Mtb-Ag levels longitudinally in children initiating TB treatment and compare among those with/without clinical improvement.

Primary analyses: We will compare mean Mtb-Ag responses between children with/without clinical improvement at enrollment (TB diagnosis, n=60), and changes between baseline, 12 and 24 weeks (treatment completion n=44) in those who initiated TB treatment using t-tests.

Exploratory analyses: We will compare mean Mtb-Ag responses at 0, 12, 24 weeks between children with confirmed/unconfirmed, and unlikely TB.

Sample size and power:
In pilot data from HIV- adults, mean ESAT-6 and CFP-10 responses decreased by 1.1nM and 1.8nM respectively with treatment over 120 days. Using conservative assumptions, we estimate 80% power to detect ~1nM in Mtb-Ag change following treatment using samples treatment initiators, but anticipate pretreatment biomarker concentration will be even higher in severely ill HIV-infected children based on pilot data with increased concentrations in HIV+ vs. HIV- adults.

Table 8 provides conservative estimates of minimum detectable mean differences for baseline and change over time between children with/without clinical improvement for various improvement proportions using pilot data standard deviation (SD) estimates of ~1.1 at any time point, and between baseline and any time point. Additional power will be gained in exploratory analyses using all enrollment (n=179), 12 (n=132), and 24 week (n=115) samples allowing for comparison between treated/untreated children.

Table 8. Minimum mean detectable differences in NanoDisk-MS detected Mtb-Ag			
	N with clinical improve-ment	Baseline min detect mean diff *N=60*	Min detect mean diff change over time *N=44*
Comb CFP-10/ ESAT-6 (nM)	10%	1.35	1.67
	30%	0.88	1.04
	50%	0.81	0.95
	70%	0.88	1.04
Assuming β=0.80, 2-sided α=0.05, SD of 1.1 at any timepoint and diff between baseline and any timepoint			

1.8   Data Collection

Specimens:
No additional samples will be collected as part of this study.

Blood Plasma and Serum: Plasma and serum is already collected and stored frozen for use in these studies. Aliquots of stored samples from the parent study will be obtained for this study. We anticipate we will use all of the aliquoted sample to run the analysis. However, any remaining samples from the aliquots will be returned and stored with the parent study samples in the study repository at the University of Washington. We will store these in accordance with with University of Washington and and KNH-UON ERC guidelines and will seek approval before using these for any other work.

Clinical data:
***No additional data will be collected as part of this study.***

The PUSH study collected detailed longitudinal data on CD4s, HIV VL, ARV treatments, clinical symptoms, and development. Children were systematically screened for TB symptoms and exposure, and sputum or gastric aspirates for Xpert and culture, urine for LAM, and stool for Xpert collected irrespective of TB symptoms.[26] CXR were read by a radiologist using

standardized forms.[44] Children were categorized as confirmed, unconfirmed, or unlikely TB based on international consensus.[43] Diagnostic results were available to study clinicians; TB treatment was initiated per Kenyan guidelines.[45] These data will be available for analysis for this study.

1.9   Planned Assays

NanoDisk-MS We propose to evaluate PUSH cohort cryopreserved samples using NanoDisk-MS which rapidly identifies and quantifies Mtb-specific Ag peptide fragment concentrations (CFP-10/ESAT-6) in blood (**Fig. 8**).[13] All clinical specimens will be handled using universal precautions by staff trained in Good Laboratory Practices. CFP-10 and ESAT-6 are actively secreted in blood of individuals with TB, detectable early in infection, and associated with Mtb pathogenesis, virulence, and macrophage response modulation.[51,52]

Blood samples (serum or plasma) undergo microwave-assisted tryptic digestion, disrupting protein complexes and releasing targeted peptides that may be undetectable in other current immunoassays targeting intact Mtb proteins. Digested samples with target peptides are mixed with antibody-labeled and energy-focusing porous discoidal nanoparticles (nanodisks). Nanodisks are enriched with silica particles which allow for precise control
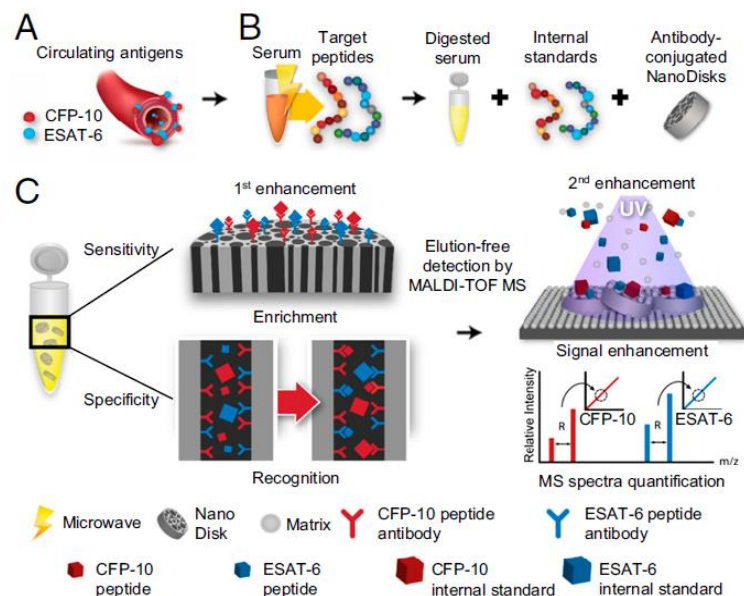
Fig. 8. NanoDisk-MS platform. A) CFP-10/ESAT-6 in circulation during active TB. B) Serum (or plasma) samples are subjected to microwave-assisted tryptic digestion and mixed with nanodisks and stable isotope-labeled internal standard peptides. C) Peptide quantification. Step 1: Recognition and enrichment of target peptides and stable isotope-labeled internal standard peptides by antibody-conjugated nanodisks. Step 2: Nanodisk enhanced MALDI signal allows target peptide quantification at low concentrations, as determined by MS intensity ratio of target and isotope labeled internal standard peptides.
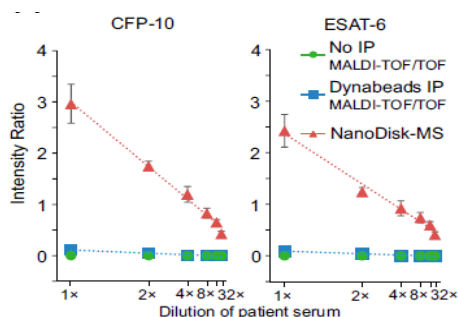
Fig. 9: Mtb-Ag MS intensity ratios at differing serum concentrations from a TB case by MALDI-TOF/TOF MS without immunoprecipitation (IP) enrichment, with Dynabead IP enrichment, and NanoDisk-MS.

of porosity, surface area and absorption properties, acting as co-matrixes for matrix-assisted laser desorption/ionization (MALDI) of bound peptides to enhance detection by high-throughput MALDI time-of-flight mass spectrometry (MALDI-TOF MS). Nanodisks are further epoxy-modified and conjugated with Mtb specific antibodies creating a high affinity, high capacity peptide enrichment platform. Calibration curves for Mtb-Ag quantification were generated plotting MS spectra using Mtb-free human serum spiked with recombinant CFP-10/ESAT-6 standards.

NanoDisk-MS detected Mtb-Ag at a wide range of sample dilutions (**Fig. 9**). Conversely, MALDI-TOF/TOF MS without immunoprecipitation (IP) failed to detect targeted peptides due to serum sodium and lipids, and only weakly detected targets after conventional IP with addition of peptide-specific Dynabeads, lost after 2x dilution. In contrast, NanoDisk-MS detected Mtb-Ag to 32x dilution, demonstrating sensitivity in

patients with low biomarker levels. Limits of quantification and detection (CFP-10: 200 pM, 50pM; ESAT-6: 650 pM. 200pM) were established based on maximum Youden index value in development cohorts of 25 HIV-negative TB cases and 25 controls.

E. ETHICS/PROTECTION OF HUMAN SUBJECTS

1.10 Potential Risks and Benefits

1.10.1 Potential Risks

The use of archived specimens involves no direct interaction with human subjects. This study does not require any additional specimen collection, laboratory procedures or medical visits and therefore does not place the original study participants at any additional physical health risk.

Confidentiality: The study team will not collect any new clinical data or have access to original study files for previously accrued cohorts and will use only cleaned already collected data from archived study databases. This data contains only coded de-identified data.

1.10.2 Potential Benefits
Direct benefits
All studies are retrospective on archived samples, and thus, there is no direct benefit to the participants of these cohort studies.

Indirect benefits
The study will add to our knowledge regarding the diagnosis of TB one of the leading causes of morbidity and mortality among HIV-infected children globally.

Importance of knowledge to be gained: Understanding the role of Mtb antigen detection for TB diagnosis and treatment monitoring using NanoDisk-MS technology can inform potential interventions for the diagnosis and improved management of TB disease in HIV-infected children. Data generated as a result of these proposed studies will be disseminated to the scientific community through the use of meeting abstracts and manuscripts submitted for publication. The results of this study may inform diagnostic guidelines, and a greater understanding of TB pathogenesis in HIV-infected infants and children.

1.11 Informed Consent Process

For the archived PUSH cohort all archived cohorts included here, HIV-infected ART-naïve hospitalized children ≤12 years and meeting study criteria were invited to enroll in the parent study.

Informed consent for for participation of the children in the research studies was obtained from caregivers of all study participants in the parent study. During their enrollment visit, clinical staff read through consent forms with the caregiver of the potential participant in their choice of English, Kiswahili, or Luo Caregivers provided written informed consent to participate in the parent study.

1.12   Participant Confidentiality

All specimens and data have been collected, and all specimens and data are de-identified.

F. DATA HANDLING AND RECORD KEEPING

1.13   Data Management

Clinical data and specimens from previously accrued cohorts have already been collected from participants and no new research procedures will be carried out on any study participants from these cohorts. Archived samples already collected from participants will be used for TB assays.

The previously collected de-identified data is stored in a REDCap database. No patient identifiers are stored in electronic form. REDCap is a secure online password protected and encrypted web application for building and managing online surveys and databases sponsored by UW Institute of Translational Health Science (https://www.iths.org/investigators/services/bmi/redcap/). REDCap is specifically geared to support online or offline data capture for research studies and operations. Data stored in REDCap is accessed through password protected and encrypted logins, and is only available to study-related investigators. The software provides 1) an intuitive interface for validated data entry; 2) automated export procedures for seamless data downloads; and 3) procedures for importing data from external sources.

All data for this study will be under the custody of the Principal Investigator, and authorized study staff. Data retrieval procedures will be similar for all types of data in this study. Authorized study staff members will download the datasets from the secure servers for routine quality checking and analyses. All downloaded data will be maintained on a secured, password-protected study computer.

1.14   Types of Data

Clincal data, including morbidity and mortality data from children was collected previously and will be available for analyses..

1.15   Study Records Retention

Data from the previously accrued cohorts has previously been collected and cleaned by a trained data team. De-identified data reside in study databases.

G. PUBLICATION/DATA SHARING POLICY

All publications will be jointly presented by UW, University of Arizona, UON and KNH collaborators, with authorship agreed upon by Drs. LaCourse, Hu, John-Stewart, and Wamalwa.

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APPENDICES

Appendix A OVERALL STRUCTURE OF STUDY TEAM
Appendix B BUDGET
Appendix C CONSENT FROM PARENT STUDY

APPENDIX A Overall structure of study team

Background and Overview

The study Principal Investigator will be responsible for general leadership, and co-investigators and other collaborators provide interdisciplinary strengths in their various areas of expertise. Sylvia LaCourse (PI) will lead the University of Washington team who will focus on the coordination of the sample repository and clinical data, as well as analyses involving clinical characteristics of the cohorts with input from Kenyan collaborators with additional relevant pediatric HIV/TB expertise. Ye Hu (Co-investigator) will lead the Arizona State University-based team who will focus on performing the lab-based assays. All teams will collaborate to perform data analyses.

Principal Investigators

The study PI was responsible for the conception of the proposed aims, developing the study design, and the grant proposal development. Throughout the proposed study, they will provide scientific leadership and oversee development of the protocol, recruitment of personnel, management of the investigative and coordinating teams, and management of sub-contracts.

University of Washington	Arizona State University	University of Nairobi
Sylvia LaCourse (PI) Grace John-Stewart (Co-I, PI of completed parent cohort (PUSH study)) Lurdes Inoue (Co-I, Bayesian latent class analysis) Staff statistician TBN Research coordinator Lab tech	Hu Lab Ye Hu (Co-I) Jian Fan (Co-I)	UW/University of Nairobi Collaborators Dalton Wamalwa (Site PI of completed parent cohort (PUSH study), peds HIV) Elizabeth Maleche-Obimbo (peds TB)

The PI, along with the co-investigators, will be responsible for analyzing, interpreting, and disseminating results.

Co-investigators and Collaborators

Drawing from their respective fields of expertise, our multi-disciplinary team of co-investigators and study collaborators were involved in the study design and grant proposal development. They will provide oversight on the study implementation and data collection procedures, as well as data analysis and interpretation of results. Co-investigators will stay updated on study proceedings through weekly data reports and quarterly meetings on overall study progress and developments.

<u>UW</u>

Dr. LaCourse (UW) will oversee the processes to use specimens and clinical data from the completed PUSH cohort for the proposed studies, coordination of analyses, and contribute to design of studies from this repository. Dr. LaCourse will oversee the maintenance and applications for regulatory ongoing cohorts and will supervise the Research Coordinator and Lab tech for their activities associated with the proposed study. The Research coordinator will manage the sample repository and with aid of the Lab tech who will prepare samples or shipment, will coordinate shipments from the clinical sites and University of Washington and Arizona State University. In coordination with the administrative team they will manage submissions of ethical approval documents.

The UW staff statistician will provide input on design of statistical analyses as well as clinical data management of the repository cohort. Support and supervision will be provided by the

UW CFAR biometric core for biostatistical analyses. Dr. Inoue will provide input on design of Bayesian latent class analyses (Aim 1 Exploratory).

<u>University of Nairobi Collaborators</u>
Dr. Wamalwa will provide guidance on HIV clinical data from the PUSH cohort. Dr. Maleche-Obimbo will provide expertise in pediatric TB clinical data. They will review clinical data at least quarterly, or more if needed. Drs. Wamalwa and Maleche-Obimbo are based at the University of Nairobi and have adjunct appointments to the University of Washington Department of Global Health.

<u>Arizona State University</u>
Dr. Hu will lead the the design, implementation and analysis of NanoDisk-MS studies. Dr. Fan will carry out the NanoDisk-MS assays on cryopreserved plasma and serum in the Hu laboratory. She will also contribute to the fractionation of the serum samples with Nanodisk, as well as the mass spectrometric analysis on MALDI-TOF/TOF and data analysis and perform Liquid Chromatography (LC)-MS (/MS) to further verify identification and sequencing of the TB biomarkers. In the proposed study, under Dr. Hu's supervision, Dr. Fan will help to design and develop the protocol of quantifying peptide signatures of TB from serum/plasma samples of patients, by combining the advanced techniques of nanosensing and absolute quantification proteomics. Drs. Hu and Fan will meet weekly to review study progress and interim analysis results.

Administrative staff
The primary administration of the award will occur at University of Washington. Administrative staff will be responsible for pre- and post-award financial administration as well as the organization and storage of all regulatory related documents. Staff will also be available at Arizona State University to assist with administration and logistical tasks to support the study.

APPENDIX B Budget

	Year 1	Year 2	TOTAL
Personnel	47,931	64,258	112,190
Benefits	14,179	18,227	32,406
Staff Subtotal	62,110	82,485	144,595
Travel	1,546	4,075	5,621
Other (Dissemination, shipping)	1,500	2,500	4,000
ASU Subcontract direct costs (includes personnel, materials and supplies, other services)	59,841	101,945	161,786
Direct Costs	124,997	191,006	316,002
Subcontract IDC	*34,109*	*58,109*	
UW IDC	50,037	49,428	99,465
TOTAL COSTS	**209,143**	**298,543**	**507,686**

Budget Justification

UNIVERSITY OF WASHINGTON

University of Washington Personnel

Sylvia LaCourse, MD, MPH (PI), 1.56 calendar months (13%) FTE to oversee this project in Y 1, and 2.28 calendar months in Y 2 (19% FTE) in Y 2.

Grace John-Stewart, MD, MPH, PhD (Co-I),. FTE 0.24 calendar months (2%) in Y 1 and 2.

Staff statistician, BS, MS, FTE is requested for 1.2 calendar months (3% FTE) in Y 1 and 2

Lurdes Inoue, MS, PhD FTE 0.6 calendar months (5% FTE) in Y 2.

Lab tech TBD FTE 0.6 calendar months (5% FTE) in Y 1

Research Coordinator (post doc) TBD, FTE 3 calendar months (25% FTE) in Y 1 and 2.

Collaborators

Dalton Wamalwa MBChB, MMed, MPH (collaborator), FTE 0.18 calendar months (1.5% FTE) in Y 1 and 2.

Elizabeth Maleche-Obimbo MBChB, MMed, MPH (collaborator), FTE 0.18 calendar months (1.5% FTE) in Y 1 and 2.

Travel

Attendance scientific meetings, including conference registration (for AIDS, International Union TB and Lung Disease or similar) for roundtrip international airfare, hotel and for per diem for a total of $1,546 for year 1 and $2,500 for year 2.

Other

University of Washington Other

Sample shipment in year 1 for $2,000, and $2,000 in year 2 for dissemination costs.

ARIZONA STATE UNIVERSITY SUBCONTRACT

ASU Senior/Key Personnel

Ye Hu, Ph.D., Co-Investigator 0.6 calendar months in all years.
Jia Fan, M.D., Ph.D., Assistant Research Professor 4.8 calendar months in all years.
<u>**Other Direct Costs**</u>

Materials and Supplies
Funds are requested for materials and supplies in the amount of $10,000 for each year:
Silicon Wafers for Nanodisk fabrication $3,000 for each year
Isolation reagents for serum sample handling $3,000 per year
Antibody for enriching target peptides $2,000 is requested per year
General lab supplies

$2,000 per year for general lab supplies including some miscellaneous chemical supplies estimated based on experience, which will be $1,000/year.
Other Services
Core Facilities Fees
Funds are requested in the amount of $2,000 per year of the project for use of ASU's core facilities.
Mass Spec Usage for Protein Identification
We request $8,000 per year for mass spec usage.

APPENDIX C Consent

(Please see attached consent)