Previous application title: Immunoglobulins in breastmilk of mothers living with HIV and infant microbiota and immunity

Date submitted: 28 February, 2020

Major changes from the prior application:

I appreciate the Reviewers' thoughtful consideration of our submission, as well as their evaluation that the proposal is highly innovative and novel, led by a strong candidate and excellent mentorship team. Major revisions in response to reviewers' comments include changes in specific aims, with clearer measurables and a stronger analytical plan. The hypotheses are now clear and can be upheld or rejected based on specific variables being measured between the groups. Extensive consultation with the CFAR Biostatistics core has been undertaken and their input included. New preliminary data is presented, included data on human breast milk immunoglobulins as well as antibody-microbe binding data from murine models. Expertise in breast milk immunology has been added to the team. Finally, under every aim, I have included a section on "Expected outcomes and alternative approaches".

Response to reviewers:

Lack of expertise in breast milk immunology among my mentors: Mentors now include [redacted], who has expertise in humoral immunity of the neonate and antibody-microbe binding in breast milk. We additionally replaced [redacted] with [redacted], who is part of both the Duke CFAR Immunology and Developmental Cores. [redacted] studies antibody responses in children infected with or infected by HIV.

Lack of clear outcomes to refute/support hypothesis: I have changed all hypotheses to make them clearly measurable. Analytic plans have been added that clearly describe how specific variables will be measured and methods that will be used to assess. The outcome of the analysis for each aim will now be sufficient to reject or support the hypotheses.

Consulting the CFAR Biostatistics core for data integration/ justification for sample size/ analytical; plan: In line with the reviewers' comments, I consulted with the CFAR Biostatistics core, including [redacted], but also [redacted] who specializes in microbiome data analytical models. They have reviewed this proposal and discussed it with me. We now clearly state how both simple and multiple linear regressions will be used to test associations between various variables. Furthermore, clear justifications for sample sizes are provided.

Experimental plan not clearly described/ cohort details missing/methods not clear: This is a substudy that will make use of samples from an ongoing parent study. I have added a brief description of the parent study and referenced relevant papers with cohort characteristics. For this substudy, the eligibility criteria for the selected women and their infants are strict, including lack of antibiotics, illness, co-infections, mastitis. Samples to be used are clearly stated; Breast milk is obtained via manual pumping with clean hands into sterile conical tubes. The first 10mL of breast milk is collected. Justification for the 4-week time point for this substudy is clearly stated. Although longitudinal data would be ideal, this will be reserved for a follow-up study on an R01. In addition,I have clarified the various methods to be used for each aim. For aim 2, we will assess antibody concentrations in stool by ELISA and antibody binding using magnetized columns and 16S sequencing. Aim 3, we will measure concentrations of 7 different immune markers in infant plasma by ELISA or luminex. I have included these clarifications in text.

Key aspects regarding breast milk not considered: Extensive text regarding previous work on immunoglobulins in breast milk have been added. Preliminary data on immunoglobulins in breast milk of women from different geographical areas is now included.

Not clear how the data might be useful in future: The following has been added; "Should we find that maternal HIV leads to altered antibody-microbe binding profile and that this associates with immune activation, this could lead to interventions such as pre- and probiotics which could be used to improve health in HIV-exposed infants."

The connection between the primary hypotheses is not well defined: I have clarified in text and included succinct hypotheses for each aim. Together the three hypotheses now align with the overall hypothesis of the study.

Specific aim 1a unlikely to be informative as total Ig likely to be driven by chronic HIV Infection: We agree that HIV infection likely drives the concentration of IgG and its subclasses in breast milk. While total antibody profile has been assessed in other secretory fluids in women living with HIV, this is not the case in breast milk. Furthermore, there is little information on levels of antibody subclasses in breast milk. Also, total IgG concentrations in breast milk likely has an influence on antibody functionality in infant gut. It's therefore informative to assess IgG and subclass concentration in breast milk considering the overall implications.

Lifelong antiretroviral treatment of pregnant mothers living with HIV has led to a decrease in vertical transmission of HIV[1]. However, this has led to a growing population of infants who are HIV-exposed and uninfected (iHEU). These infants have up to four-fold higher rates of morbidity and mortality from infections compared to unexposed infants[2–4], possibly due to their altered immunological profiles[5] and heightened immune activation[6,7]. The mechanisms behind their elevated immune activation are largely unknown. Among the factors that have been suggested to impact immune development in the iHEU infants include the developing gut microbiota.

Human milk provides direct immunity through transfer of immune components from mother to offspring[8]. Among the main immune factors transferred in breast milk are immunoglobulins (Igs) which are important effectors of the adaptive immune system[9]. Previous work in murine models has shown that immunoglobulins in breast milk (murine IgG3, IgG2b and IgA) dampen mucosal T cell responses in the offspring through binding of commensal gut bacteria; thus limiting inflammatory immune responses to commensals[10]. Importantly, these IgG subclasses are able to induce T independent (TI) responses and are less inflammatory than other subclasses[10,11].

Persons living with HIV (PLWHIV) have altered gut microbiota[12,13], and multiple antibody abnormalities[14–16], but whether HIV infection impacts total immunoglobulin levels and subclass distribution in breast milk as well as commensal binding capability in offspring is unknown. Further, it is unclear whether microbe-antibody interaction in the infant gut is involved either directly or indirectly in mediating immune activation in human infants. This proposal seeks to address this knowledge gap to determine (1) whether maternal HIV infection impacts total immunoglobulin and subclass distribution in breast milk, (2) whether maternal HIV infection impacts antibody concentration and antibody-microbe binding profile in infant gut and (3) whether immunoglobulin-microbe interactions in the gut of iHEU correlate with immune activation. We hypothesize that HIV infection alters the quantity and subclass distribution of total antibody in breast milk which impacts antibody-microbe binding profile in the infant gut. This in turn shapes infant gut microbiota resulting in increased immune activation. ***Findings will provide critical insights into the maternal imprinting of iHEU and will form preliminary data for a K43 application for [redacted].***

Aim 1: Compare concentrations of immunoglobulin isotypes and subclasses in breast milk of women living with HIV (WLHIV) versus uninfected mothers at 4 weeks postpartum. We will measure total levels of various immunoglobulin isotypes and their subclasses in breast milk by ELISA.

Aim 2: Compare the immunoglobulin concentrations and immunoglobulin-microbe binding profile of IgG and IgA subclasses in stool of iHEU versus iHU infants at 4 weeks of age. Pelleted stool bacterial cells will be stained with fluorochrome conjugated IgG or IgA subclasses and positively selected. Bound and unbound bacteria will be subjected to 16S microbial sequencing to determine taxonomy of bacteria. Antibody concentrations will be determined as in Aim 1.

Aim 3: Assess the relationship between antibody-microbe binding and immune activation in infants. Markers of immune activation (sCD163, IL-6, IL-8, IFN-γ, TNF-α) and microbial translocation (iFABP, LBP) will be measured in infant plasma and correlated with immunoglobulin bound bacteria in infant gut.

SIGNIFICANCE

Over 3.5 million women of childbearing age are infected annually with Human Immunodeficiency Virus type 1 (HIV-1**)** in sub Saharan Africa resulting in large numbers of children being HIV-exposed[16]**.** Implementation of successful prevention of vertical transmission programs have reduced rates of transmission to as low as 1% with the use of combination antiretroviral therapy (cART) during pregnancy[15]. However, this results in approximately 1.6 million infants who are HIV-exposed but uninfected (iHEU) delivered annually. iHEU display multiple immune alterations, and elevated immune activation, including heightened T cell and monocyte activation[6,7]. We and others have also found that iHEU also have altered gut microbiota ([17], **Fig 1**), with lower gut bacterial (alpha) diversity through the first 15 weeks of life**.** It is however not clear what factors contribute to the altered microbiota or immunity in these infants. One mechanism that remains underexplored is the role of maternal breast milk in shaping microbiota and immunity in infants.

Human milk is composed of a plethora of immunologic factors that protect the infant against infections[8]. Among the transferred factors are maternal antibodies which help protect mucosal membranes in neonates in concert with other breast milk factors such as lactoferrins[18]. Though a risk factor for HIV transmission, breastfeeding is recommended by the WHO due to the high morbidity rates associated with replacement feeding. In addition to helping protect infants from infection, breast milk antibodies also help regulate host-microbiota interactions in infants. We (Koch lab) showed that breast milk-acquired maternal antibodies bind to commensal bacteria in the offspring gut and help ensure tolerance to these commensals[10]. Murine pups who did not receive passive

antibody had exaggerated intestinal T follicular helper cell and germinal center B cell responses, and had elevated levels of serum inflammatory cytokines following induction of intestinal inflammation[10]. In addition, antibody-microbe interactions have also been shown to regulate systemic inflammation in humans[19]. Serum anticommensal-IgG had an inverse correlation with plasma level of CD14 (marker of monocyte activation) in both healthy donors and patients with IgA-deficiency indicating that systemic anti-microbiota responses correlate with reduced inflammation[19]. It is not unclear whether the altered pool of antibodies transferred to offspring in humans will have a similar immune-regulatory role or contribute to systemic inflammation based on interaction with the underlying microbiota.

In humans, there are two antibody isotypes that are transmitted via breast milk: IgA and IgG[20]. Immunoglobulins in breast milk arise from systemic or local sources and depend on history of antigenic exposure of the mother[21]. Within IgG and IgA, there are additional subclasses: IgG1-IgG4 and IgA1-IgA2 respectively[22]. Each antibody isotype and subclass play distinct roles in the immune response based on the kinetics in which they appear, as well as their ability to interact with both the antigen and the innate immune system[22]. Importantly, IgG2 in humans is dominant in responses to T-independent antigens[23], including to most commensal bacteria[19]. IgG2 is known to have reduced inflammatory capacity relative to IgG1 and makes up a large proportion of the mucosal IgG pool in health[24], but not in some inflammatory conditions such as ulcerative colitis[25].

Chronic HIV infection leads to abnormalities in both quality and quantity of antibody[15,16]. Persons living with HIV (PLWHIV) have abnormal B cell compartment and defects in antibody function[14], including abnormalities in antibody production[26,27] and skewed immunoglobulin subclass distribution in their serum and other fluids[28]. Of note, IgG2, the subclass known for its less inflammatory, T-independent responses in humans, is deficient in plasma of PLWHIV[28] and iHEU[29]. HIV appears to alter IgG subclass transplacental transfer efficiency[30], yet to date, few studies have investigated subclass distribution transferred via breast milk to iHEU[31]. It is feasible that maternal HIV infection impacts the quantity, subclass distribution and/ or binding profile of total antibody in breast milk leading to differential transfer and programming of the gut microbiota and consequent immune activation in the infant. This proposal seeks to test this by investigating (1) whether maternal HIV impacts total levels of antibody and their subclasses in breast milk and infant gut, (2) whether maternal HIV alters the immunoglobulin-microbe binding profile and (3) whether the impact of maternal HIV-1 infection on transferred immunoglobulin subclasses and antibody-microbe binding in the infant gut correlates with immune activation in infants. These Aims will lead to better understanding of how breast milk contributes to shaping early immune development in iHEU.

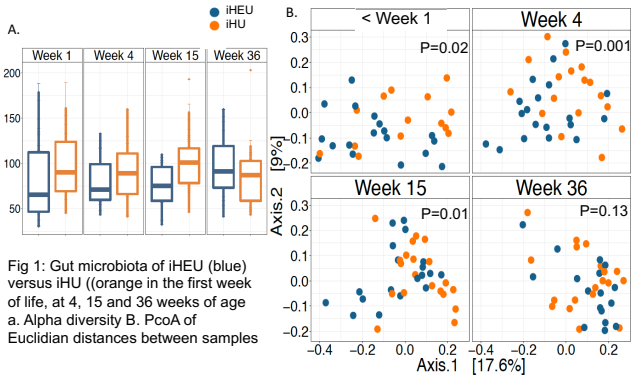


Fig 1: Gut microbiota of iHEU (blue) versus iHU ((orange in the first week of life, at 4, 15 and 36 weeks of age a. Alpha diversity B. PcoA of Euclidian distances between samples

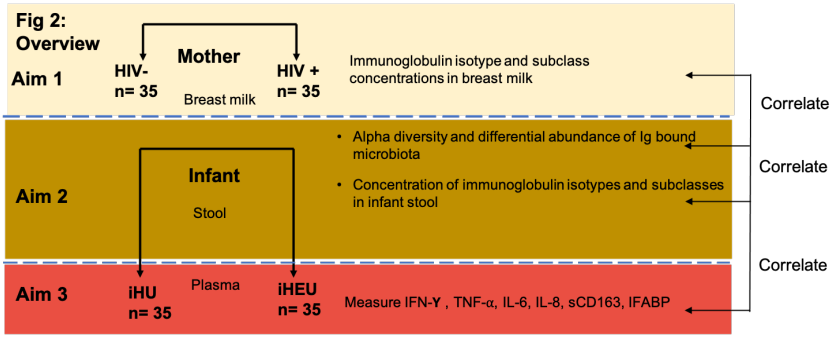
INNOVATION

The work proposed here will address a research question of enormous scientific and public health significance, and is highly innovative. While it is clear that iHEU infants have increased inflammation, high infectious disease morbidity and altered intestinal microbiota, the mechanisms responsible for these phenotypes are largely unknown. We propose a highly innovative approach to testing the distribution of antibody subclasses in breast milk that are transferred to infants and role of breast milk in differentially shaping both microbiota and immunity in these infants. Here, we have asked relevant questions pertaining to the role of breast milk antibodies in shaping infant gut microbiota and mediating immune activation that thus far remain unanswered. Moreover, while the effect of antibody-microbe binding on mucosal tolerance has been tested in murine models[10,32], this has not been done in humans. In addition, while anti-commensal IgG2b and IgG3 subclasses that are known to be T-independent have been described in murine models, it is unclear whether humans mount similar subclass responses to their intestinal microbiota especially in iHEU who have reduced IgG2[29]. Our unique cohort will afford us an opportunity to address these questions and test the impact of breast milk immunoglobulins and microbe binding on immune activation in infants.

APPROACH

Cohort: Samples from WLHIV (n=35) and uninfected women (n=35) and their infants who enrolled in the InFANT study, which has been recruiting mother-infant pairs at the Midwife Obstetric Unit (MOU) in Khayelitsha, Cape Town, South Africa since 2014, will be utilized[33,34]. Sufficient samples for this proposed substudy have been

collected and stored. We will take advantage of this repository to study WLHIV who have exclusively breastfed for at least a month. WLHIV who will be selected for this substudy are receiving cART based on national guidelines[35], and have a CD4+ T cell count above 350 cells/ul with undetectable viral load. The WLWHIV have been matched with a control group (HIV uninfected women) by age and parity. Sociodemographics are highly cohesive in this cohort[33].



Eligible substudy participants will have had vaginal term deliveries of average for gestational age infants, have no known tuberculosis contacts, no evidence of mastitis or cracked nipples, and no antibiotic administration in the past 6 months. iHEU will have documented negative HIV DNA PCRs at birth and 4 weeks. We will use maternal (breast milk; obtained by manual pumping with clean hands into sterile tubes) and infant samples (stool and blood) collected at week 4 after delivery in this substudy. This time point was chosen to allow the study of vertical microbiota transmission prior to the introduction of Cotrimoxazole which is administered to iHEU at 6 weeks; when maternal passive immunoglobulin is still present, and when breast feeding is well established. Breast milk, stool and plasma samples are stored at -80° C. Samples will be shipped to Seattle for this proposal. A study overview is shown in **Fig 2.**

Aim 1: *Compare concentrations of immunoglobulin isotypes and subclasses in breast milk of women living with HIV (WLHIV) versus uninfected mothers at 4 weeks postpartum*

Rationale: WLHIV have polyclonal hypergammaglobulinemia that is not fully reversed by cART[36], and have deficiency of IgG2 in their plasma. Similarly, iHEU have increased intestinal mucosal levels of total IgG and IgA versus iHU, suggesting breastmilk-acquired antibody may differ[37]. While total and antigen specific antibody titers have been assessed in serum of HIV infected mothers[38,39], it is unclear how maternal HIV impacts immunoglobulin isotypes and subclasses in breast milk. We hypothesize that breast milk from WLHIV has higher concentrations of IgG, IgG1 and IgA and lower concentrations of IgG2 compared to breast milk from uninfected mothers.

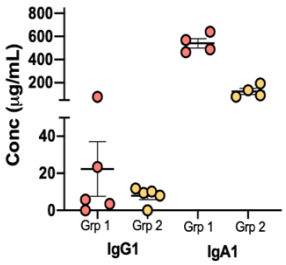


Fig 3: IgG1 and IgA1 concentrations from two groups of healthy women

Preliminary data: We have optimized an ELISA to measure total immunoglobulin subclasses in breast milk. Our preliminary data using breast milk from healthy women from two different geographical locations is consistent with literature[40] and shows IgA to be more abundant in breast milk compared to IgG (**Fig 3**: **Prelim data Aim 1**).

Breast milk: A maximum of 10ml per breast of milk has been collected from each participant. Samples are centrifuged to separate milk into cells, lipid layer and aqueous phase. We will determine total concentrations of various immunoglobulin subclasses by ELISA. We have experience performing ELISAs on breast milk samples from mice[41,42] and humans as in preliminary data (**Fig 3**), and we will use a similar approach in this proposal. Data will be analyzed by Soft Max Pro software and shown as median concentration.

Power calculations and statistical analysis: Sample size calculations were done using total IgG as the primary outcome based on concentrations in breast milk between 1 and 12 months postpartum, where the mean IgG concentration was 9.69 (±1.59 SD)[37]. With 70 mother-infant pairs (35 iHEU and 35 iHU), we anticipate that we will be able to detect a difference of at least 15% with 90% power. Statistical testing of the immunoglobulin concentrations between the groups will be done using the two-sided Mann-Whitney U test.

Expected outcomes and alternative approaches: We expect that the absolute concentrations of total IgG, IgA, IgM, as well as IgG and IgA subclass distribution will differ in breast milk of WLHIV versus uninfected women. A limitation of this study is that it is cross-sectional and the quality of breast milk changes through time. We have addressed this by choosing 4 weeks postpartum because this time point allows us to study breastmilk and infant stool microbiota when breastfeeding is well-established but prior to the introduction of cotrimoxazole administered to iHEU at 6 week.

Aim 2: *Compare the concentrations and immunoglobulin-microbe binding profile of IgG and IgA subclasses in stool of iHEU versus iHU infants at 4 weeks of age.*

Rationale: One way transferred immunoglobulin subclasses potentially influence neonates is by binding to commensal or pathogenic bacteria thus influencing intestinal microbiota in the offspring and in turn developing immunity[43,44]. Different antibody subclasses can initiate qualitatively distinct immune responses, such as tolerance or inflammation[10]. In healthy mice, the majority of the anti-commensal IgG antibodies are of IgG2b and IgG3 subclasses[10]. Murine IgG2b and IgG3 subclasses are known to bind inhibitory Fc Receptors, and to be T-independent[11]. In humans, the equivalent subclass is likely IgG2, and indeed, plasma IgG2 has been found to have high reactivity to commensal bacteria[19]. However, it is unknown whether the anti-commensal subclass binding profile will be impacted in the iHEU gut, despite skewed subclass distribution. We hypothesize that due to differences in breastmilk-acquired antibody subclass distribution and/or altered microbe coating, significantly lower number of bacterial taxa will be immunoglobulin-bound in stool of iHEU compared to iHU, specifically by IgG2.

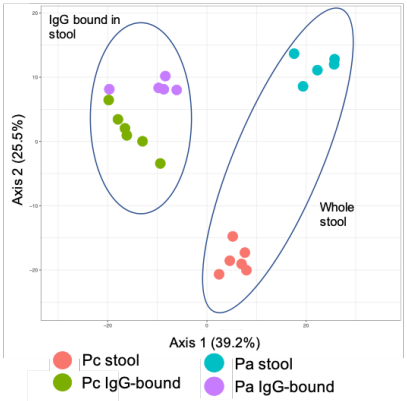


Fig 4: Principal component analyses of whole stool microbiota and IgG bound microbiota in pups stool.

Preliminary data: We altered gut microbiota and consequent total IgG levels in pregnant mice as previously described[41]. We examined IgG bound bacteria in pups' gut. Stool pellets were homogenized in PBS and centrifuged to pellet bacteria. Bacterial pellets were stained with fluorochrome conjugated anti-mouse IgG and positively selected by magnetized columns. Bacterial DNA was extracted from bound and unbound fractions and subjected to 16S rRNA gene sequencing. Fecal microbiota of pups born to altered IgG mothers (Pa) clustered distinctly from that of pups born to control mothers (Pc) by Principal Component Analysis (PCoA) (**Fig 4; prelim data Aim 2**). Furthermore, IgG-bound stool microbiota was distinct between the two groups and from total stool microbiota, suggesting that maternal antibody may influence neonatal gut composition by differential antibody-microbe binding in the neonatal gut.

Stool Immunoglobulin concentration and subclass distribution: Stool will be resuspended in PBS/protease inhibitor cocktail by vortexing. Sample dilutions will be made and antibody ELISAs performed as in Aim 1.

Magnetic separation of immunoglobulin bound and unbound bacteria in infant gut: Frozen infant stool samples will be thawed on ice, weighed and resuspended in PBS/protease inhibitor cocktail. Samples will be vortexed until resuspended, and bacterial cells pelleted. Supernatants (free immunoglobulins) will be carefully removed. Bacterial pellets will then be stained with PE or APC conjugated anti-IgA, IgG1, IgG2, IgG3 or IgG4, with Fc block to prevent nonspecific binding. Fluorochrome conjugated antibodies will then be labeled with magnetic beads. Stained bacterial cells will be passed through magnetized columns and eluted to separate immunoglobulin bound bacteria fractions (Ig+) by positive selection or unbound bacteria (Ig-) by negative selection. Bacterial DNA will be extracted from both fractions using the PowerSoil DNA extraction kit. Libraries will be prepared by amplifying the V3-V4 region of the 16SrRNA gene and sequenced on the MiSeq platform as previously described[45]. This will allow identification of bacteria in the sorted fractions and determine abundance of various taxa in the bound and unbound for the various immunoglobulins.

16S microbiome analysis: As previously described[46], barcodes and primers will be removed by cutadapt [47]. Trimmed sequences will be imported into R and processed using the DADA2 (v1.12.1) pipeline[48]. Taxonomic assignment will be performed using Silva (v132) reference database[49]. Downstream analysis will be done using phyloseq[50], alongside other R packages. Differential abundance testing will be conducted by DeSeq2[51]; the log fold change set to 1.5. Output p-values will be adjusted for multiple comparisons by Benjamini-Hochberg correction[51] and taxa with adjusted p-values <0.05 will considered statistically significant.

Power calculations and statistical analysis: Based on the data in **Fig 1**, the sample size of 35 per group will allow us to detect the same difference in alpha diversity we saw at 4 weeks of age between stools of iHEU (Chao1= 65, ± 29.6 SD) and iHU (Chao1=89.8, ± 40.7 SD) with 83% power, assuming similar differences in diversity are evident within the bound fraction of the microbiota. We will use DESeq2[51] to determine differentially abundant taxa between the groups in immunoglobulin bound bacteria. We will test for the associations of immunoglobulins concentrations in breast milk or infant stool with microbial alpha diversity (Chao1) using simple linear regression. We will similarly model the associations of these immunoglobulins with our top 10 differentially abundant bacterial taxa of the bound fraction of microbiota in the gut (present in at least 40% of all samples). In the latter models, taxon abundances will be transformed using the centered log ratio (CLR) to account for the compositional nature of microbiome data. All models will be adjusted for potential confounders and the significance of regression coefficients will be assessed using type 1 error at the 0.05 level.

Expected outcomes and alternative approaches: We expect to identify different antibody subclass distribution and immunoglobulin bound vs unbound bacterial taxa in iHEU versus iHU[10,52], and that iHEU will have lower diversity of IgG2 bound commensal bacteria compared to iHU infants. A potential pitfall is that, while we expect IgG2 in iHEU to be bound to fewer commensal bacteria than iHU, it is possible that we may observe similar patterns of bound bacteria in both groups due to differences in the underlying gut microbiota, which we will also compare. Should we find this to be the case, we will instead examine the binding profile of IgG2 from both infant groups using a common source of microbiota (stool from a healthy infant donor) as previously described[10].

Aim 3: *To assess the relationship between antibody-microbe binding and immune activation in infants*

Rationale: Studies in mice have found that maternal antibody dampens immune responses to commensal organisms[10], but whether this phenomenon occurs in humans is unknown. Microbial translocation in the gut likely leads to immune activation. iHEU have elevated immune activation and inflammation[53,54,23]. We will measure the concentration of both proinflammatory cytokines and markers of immune activation in plasma and assess whether microbial translocation is increased in iHEU. We will determine whether the immunoglobulin-microbe binding (Aim 2) in the infant gut has any association with immune activation or microbial translocation in infants. We hypothesize that, due to lack of or altered microbe coating of IgG2 to commensal bacteria in the gut, iHEU develop microbial translocation and increased immune activation compared to iHU; and that IgG2 microbe binding negatively correlates with immune activation in iHEU.

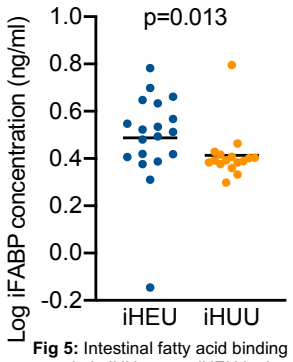


Fig 5: Intestinal fatty acid binding protein in iHU versus iHEU in the first week of life

Preliminary data: We tested the hypothesis that iHEU develop intestinal barrier dysfunction due to inadequate transmission of commensal reactive antibodies in breast milk. We measured intestinal Fatty acid binding protein (iFABP), a marker of intestinal barrier integrity, in human infant plasma in the first week of life. Indeed, iHEU had significantly higher plasma concentrations of iFABP than iHU (**Fig. 5, prelim data Aim 3**). Whether these differences in microbial translocation persist long-term is unknown.

Plasma markers of immune activation: ELISA tests for determination of sCD163, I-FABP (R&D Systems) and LBP (Hycult Biotech) will be used. We have already determined the optimal dilutions for each biomarker in infant plasma (**Fig 5**, [56]).

Cytokine quantification by multiplex assay: Cytokine concentrations in plasma will be determined by a multiplex panel containing beads for IL-6, IL-12p70, TNF-α, IL-8 and interferon (IFN)-γ (ProcartaPlex, eBioscience). Each sample will be assayed in duplicate. Intra- and inter-plate controls will be included and Spearman correlation values >0.8 will be considered acceptable for controls.

Sample size calculations and statistical analysis: Based on our preliminary data from **Fig 5** where we were able to detect differences in concentrations of iFABP between iHEU versus iHU at week 1 of life, we anticipate we will detect similar differences at week 4 with 35 infants per group. We will test associations between abundant taxa in IgG2 bound microbiota with concentrations of immune activation markers (IL-6, IL-8, IFN-γ, TNF-α, sCD163) or markers of microbial translocation (LBP, iFABP). We will begin by fitting 7 multiple linear regression models, each aimed at estimating marker associations with the top 10 differentially abundant bacterial taxa. As in Aim 2, abundances will be based on CLR transformed counts of taxa present in at least 40% of all samples and each model will be adjusted for potential confounders. This will produce 70 regression coefficients (7 models, each having 10 microbial predictors) which will be used to find patterns of microbial expression that are associated with immune activation. For this, we will form a 7x10 array (heat map) of coefficient values and use hierarchical clustering on the columns to seek out which microbial abundance profiles exhibit similar effects on these markers. Finally, we will identify the most statistically significant taxa (coefficients) in this array using a Benjamini-Hochberg correction for multiple comparisons at the level of 0.1.

Expected outcomes and alternative approaches: It is possible that immunoglobulin binding of microbes exerts its effect on inflammation at the mucosa and not via altering microbial translocation. Should we find no difference in microbial translocation, we will measure markers of mucosal inflammation (e.g. Fecal calprotectin) in infant stools to explore alternative hypotheses.

In sum, we present a highly innovative proposal that seeks to unravel the mechanisms of maternal HIV on infant immunity. Should we find that HIV infection in mothers influences immunoglobulin subclass distribution and antibody-microbe binding in the gut of iHEU resulting in immune activation; this would have broad implications that could be harnessed to improve immunity in HIV-exposed infants, such as pre- or probiotics.

1. UNAIDS. *Fact sheet-world AIDS day 2017. Geneva: UN Joint Programme on HIV/AIDS (UNAIDS); Programme on HIV/AIDS* (2017). doi:978-92-9173-945-5
2. Weinberg, A. *et al.* Factors associated with lower respiratory tract infections in HIV-exposed uninfected infants. *AIDS Res. Hum. Retroviruses* **34**, 527–535 (2018).
3. Slogrove, A. L., Cotton, M. F. & Esser, M. M. Severe infections in HIV-exposed uninfected infants: Clinical evidence of immunodeficiency. *J. Trop. Pediatr.* **56**, 75–81 (2010).
4. Shapiro, R. L. *et al.* Infant Morbidity, Mortality, and Breast Milk Immunologic Profiles among Breast-Feeding HIV-Infected and HIV-Uninfected Women in Botswana. *J. Infect. Dis.* **196**, 562–569 (2007).
5. Abu-Raya, B., Kollmann, T. R., Marchant, A. & MacGillivray, D. M. The immune system of HIV-exposed uninfected infants. *Front. Immunol.* **7**, 1–10 (2016).
6. Dirajlal-Fargo, S. *et al.* HIV-exposed-uninfected infants have increased inflammation and monocyte activation. *AIDS* **33**, 845–853 (2019).
7. Kidzeru, E. B. *et al.* In-utero exposure to maternal HIV infection alters T-cell immune responses to vaccination in HIV-uninfected infants. **19**, 161–169 (2014).
8. Lessen, R. & Kavanagh, K. Position of the Academy of Nutrition and Dietetics: Promoting and Supporting Breastfeeding. *J. Acad. Nutr. Diet.* **115**, 444–449 (2015).
9. Tha-In, T., Bayry, J., Metselaar, H. J., Kaveri, S. V. & Kwekkeboom, J. Modulation of the cellular immune system by intravenous immunoglobulin. *Trends in Immunology* (2008). doi:10.1016/j.it.2008.08.004
10. Koch, M. A. *et al.* Maternal IgG and IgA Antibodies Dampen Mucosal T Helper Cell Responses in Early Life. *Cell* **165**, 827–841 (2016).
11. Markowitz, J. S., Rogers, P. R., Grusby, M. J., Parker, D. C. & Glimcher, L. H. B lymphocyte development and activation independent of MHC class II expression. *J. Immunol.* **150**, 1223–1233 (1993).
12. Lozupone, C. A. *et al.* Alterations in the gut microbiota associated with HIV-1 infection. *Cell Host Microbe* **14**, 329–339 (2013).
13. Vujkovic-Cvijin, I. *et al.* HIV-associated gut dysbiosis is independent of sexual practice and correlates with noncommunicable diseases. *Nat. Commun.* **11**, (2020).
14. Connors, N. A. D.-R. and M. Antibody Secreting B-cells in HIV Infection. *Curr. Opin. HIV AIDS* **4**, 426–430 (2009).
15. Kardava, L. *et al.* Abnormal B cell memory subsets dominate HIV-specific responses in infected individuals. *J. Clin. Invest.* **124**, 3252–3262 (2014).
16. Moir, S. & Fauci, A. S. B cells in HIV infection and disease Susan. *Nat. Rev. Immunol.* **9**, 235–245 (2009).
17. Bender, J. M. *et al.* Maternal HIV infection influences the microbiome of HIV-uninfected infants. *Sci. Transl. Med.* **8**, 349ra100-349ra100 (2016).
18. Gregory, K. E. & Walker, W. A. Immunologic Factors in Human Milk and Disease Prevention in the Preterm Infant. *Curr. Pediatr. Rep.* **1**, 222–228 (2013).
19. Fadlallah, J. *et al.* Synergistic convergence of microbiota-specific systemic IgG and secretory IgA. *J. Allergy Clin. Immunol.* **143**, 1575-1585.e4 (2019).
20. Franklin, E. C. STRUCTURE AND FUNCTION OF IMMUNOGLOBULINS By. (1974).
21. Hurley, W. L. & Theil, P. K. Perspectives on immunoglobulins in colostrum and milk. *Nutrients* **3**, 442–474 (2011).
22. Spiegelberg, H. L. Biological Role of Different Antibody Classes. *Int Arch Allergy Appl Immunol* **90**, 22–27 (1989).
23. Amlot, P. . & Hayes, A. E. IMPAIRED HUMAN ANTIBODY RESPONSE TO THE THYMUS-INDEPENDENT ANTIGEN, DNP-FICOLL, AFTER SPLENECTOMY. 1008–1011 (1985).
24. Benckert, J. *et al.* The majority of intestinal IgA+ and IgG+ plasmablasts in the human gut are antigen-specific. *J. Clin. Invest.* **121**, 1 (2011).

25. Scott, M. G. *et al.* Spontaneous secretion of IgG subclasses by intestinal mononuclear cells: differences between ulcerative colitis, Crohn's disease, and controls. *Clin. Exp. Immunol.* **66**, 209–15 (1986).
26. D'Orsogna, L. J., Krueger, R. G., McKinnon, E. J. & French, M. A. Circulating memory B-cell subpopulations are affected differently by HIV infection and antiretroviral therapy. *Aids* **21**, 1747–1752 (2007).
27. Hart, M. *et al.* Loss of Discrete Memory B Cell Subsets Is Associated with Impaired Immunization Responses in HIV-1 Infection and May Be a Risk Factor for Invasive Pneumococcal Disease. *J. Immunol.* **178**, 8212–8220 (2007).
28. Raux, M. *et al.* IgG subclass distribution in serum and various mucosal fluids of HIV type 1-infected subjects. *AIDS Res. Hum. Retroviruses* **16**, 583–594 (2000).
29. Baroncelli, S. *et al.* Dynamics of immunoglobulin G subclasses during the first two years of life in Malawian infants born to HIV-positive mothers. *BMC Pediatr.* **20**, 1–9 (2020).
30. Martinez, D. R. *et al.* Fc Characteristics Mediate Selective Placental Transfer of IgG in HIV-Infected Women. *Cell* **178**, 190-201.e11 (2019).
31. Gaensbauer, J. T. *et al.* Impaired Haemophilus influenzae type b transplacental antibody transmission and declining antibody avidity through the first year of life represent potential vulnerabilities for HIV-exposed but-uninfected infants. *Clin. Vaccine Immunol.* **21**, 1661–1667 (2014).
32. Wilmore, J. R. *et al.* Commensal Microbes Induce Serum IgA Responses that Protect against Polymicrobial Sepsis. *Cell Host Microbe* **23**, 302-311.e3 (2018).
33. Tchakoute, C. T. *et al.* Breastfeeding mitigates the effects of maternal HIV on infant infectious morbidity in the Option B+ era. *Aids* 1 (2018). doi:10.1097/QAD.0000000000001974
34. Kiravu, A. *et al.* Bacille Calmette-Guérin Vaccine Strain Modulates the Ontogeny of Both Mycobacterial-Specific and Heterologous T Cell Immunity to Vaccination in Infants. *Front. Immunol.* **10**, 1–11 (2019).
35. Meintjes, G. *et al.* Adult antiretroviral therapy guidelines 2017. *South. Afr. J. HIV Med.* **18**, 1–24 (2017).
36. Baroncelli, S. *et al.* Immune Activation and Microbial Translocation Markers in HIV-Exposed Uninfected Malawian Infants in the First Year of Life. *J. Trop. Pediatr.* **65**, 617–625 (2019).
37. Moussa, S. *et al.* Adaptive HIV-Specific B Cell-Derived Humoral Immune Defenses of the Intestinal Mucosa in Children Exposed to HIV via Breast-Feeding. *PLoS One* **8**, (2013).
38. De Moraes-Pinto, M. I. *et al.* Placental transfer and maternally acquired neonatal IgG immunity in human immunodeficiency virus infection. *J. Infect. Dis.* **173**, 1077–1084 (1996).
39. Jones, C. E. *et al.* Maternal HIV infection and antibody responses against vaccine-preventable diseases in uninfected infants. *JAMA - J. Am. Med. Assoc.* **305**, 576–584 (2011).
40. Czosnykowska-Łukacka, M., Lis-Kuberka, J., Królak-Olejnik, B. & Orczyk-Pawiłowicz, M. Changes in Human Milk Immunoglobulin Profile During Prolonged Lactation. *Front. Pediatr.* **8**, 1–12 (2020).
41. Nyangahu, D. D. *et al.* Disruption of maternal gut microbiota during gestation alters offspring microbiota and immunity. *Microbiome* **6**, 1–10 (2018).
42. Darby, M. G. *et al.* Pre-conception maternal helminth infection transfers via nursing long-lasting cellular immunity against helminths to offspring. *Sci. Adv.* **5**, 1–10 (2019).
43. Cullender, T. C. *et al.* Innate and adaptive immunity interact to quench microbiome flagellar motility in the gut. **14**, 571–581 (2013).
44. Kawamoto, S. *et al.* Foxp3+ T Cells Regulate Immunoglobulin A Selection and Facilitate Diversification of Bacterial Species Responsible for Immune Homeostasis. *Immunity* **41**, 152–165 (2014).
45. Gohl, D. M. *et al.* Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. *Nat. Biotechnol.* **34**, 942–949 (2016).
46. Nyangahu, D. D. *et al.* Preconception helminth infection alters offspring microbiota and immune subsets in a mouse model. *Parasite Immunol.* e12721 (2020). doi:10.1111/pim.12721
47. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* **17**, 10–12 (2011).
48. Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W. & A, A. J. Dada2. *Nat Methods* **13**, 581–583

(2016).

49. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **41**, 590–596 (2013).
50. McMurdie, P. J. & Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* **8**, e61217 (2013).
51. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).
52. Bunker, J. J. *et al.* Innate and Adaptive Humoral Responses Coat Distinct Commensal Bacteria with Immunoglobulin A. *Immunity* **43**, 541–553 (2015).
53. DIRAJLAL-FARGO, S. *et al.* HIV-exposed uninfected infants have increased inflammation and monocyte activation. *AIDS* **176**, 139–148 (2019).
54. Lohman-Payne, B. *et al.* HIV-exposed uninfected infants: elevated cord blood Interleukin 8 (IL-8) is significantly associated with maternal HIV infection and systemic IL-8 in a Kenyan cohort. *Clin. Transl. Med.* **7**, 26 (2018).
55. Schoeman, J. C. *et al.* Fetal metabolic stress disrupts immune homeostasis and induces proinflammatory responses in human immunodeficiency virus type 1-And combination antiretroviral therapy-exposed infants. *J. Infect. Dis.* **216**, 436–446 (2017).
56. Gasper, M. A. *et al.* BCG vaccination induces HIV target cell activation in HIV-exposed infants in a randomized trial. *JCI insight* **2**, e91963 (2017).

PROTECTION OF HUMAN SUBJECTS.

The main goals of this proposal are to understand better if immunoglobulins in the breast milk of mothers living with HIV contribute to colonization of their infants with an aberrant microbiome as well as increased immune activation. The research proposed will make use of stored samples generated from an ongoing study in Khayelitsha, Cape Town (PIs Jaspan/ Clive Gray). The study, entitled Innate Factors Associated with Nursing Transmission (InFANT), is enrolling a total of 550 mother-infant pairs at the Cape Town site, of which 300 are HIV-exposed and 250 HIV-unexposed. As of September 2020, 538 mother-infant pairs have been enrolled, providing far more than needed for the proposed study. For this study, infants were enrolled upon delivery and maternal history, birth history and samples were obtained. Follow-up visits were conducted at multiple time points, (week 4 relevant for this proposal), with extensive questionnaires, anthropometrics, examinations, and sample collections (blood, stool, saliva, vaginal and breastmilk) performed at each visit. The bacterial microbiota is being assessed in breastmilk and infant stool as a part of U01AI131302 (MPI Blish, Jaspan, Gray) and AI120714-01A1 (PI Jaspan). However, the InFANT study does not examine the role of antibody acquired in breast milk in shaping both the infant gut and how this relates to immune tolerance **Thus, for the proposed study, we will use the existing infrastructure and sample breadth of the ongoing, funded, observational InFANT study to perform this research.**

Dr Nyangahu's primary appointment is at Seattle Children's Research Institute (SCRI) as a postdoctoral research fellow and he is currently in the process of being promoted to a Research Scientist at the Center for Global Infectious Disease Research.

Sources of Material. For this study, we will use stored breast milk, plasma and stool samples from mothers and infants. **All samples have already been collected and stored as part of the ongoing InFANT study.** For the present study, all of the human DNA extraction and clinical immunology lab work (ELISAs) will take place at SCRI. All samples required for the present study will be shipped to SCRI.

Potential risks. There are no additional samples being collected for this substudy. In the parent study, there were minimal risks associated with blood collection, including anemia, pain, bruising and infection at the site of blood draws. This volume and frequency of the blood collection schedule complies with both South African, US and international safety guidelines.

The parent study consent process made it clear to participants that the choice to participate in the study was entirely voluntary and that acceptable alternatives included not participating or withdrawing from the study at any time. It was further explained that her choice would not affect in any way affect the routine care that she or her infant would receive from the clinic.

Protection against risk.

1. Confidentiality and Procedure Risks. A variety of mechanisms have been established to protect the confidentiality of medical records and data procured in this research. All study staff members have signed or will sign a pledge of confidentiality. Databases are password protected; a unique study ID number was assigned to participants at the beginning of the study and this number will be used as the indirect identifier in databases and lab specimens. We will continue to ensure that privacy is maintained. All data will be kept confidential, coded and kept under lock and key. Databases are password protected, and will remain as such. Results of immunological testing will not be made available to the participants.

2. Adverse Events. While there have currently been no reported adverse events related to sample collection in the InFANT study, clinical study staff in Cape Town was and remains poised to provide any care related to harm from sampling. The University of Cape Town is insured for protection against any study-related harms.

For the InFANT study, prompt reporting of any unexpected adverse experiences to each institution's IRB was expected. Any adverse events were recorded through the adverse experience report (AER) and communicated to the relevant individuals within the period of time dictated by government regulations.

3. Data and Safety Monitoring Plan. The proposed project is a sub-study of an observational study that does not warrant a separate DSMB. As such, the investigators will submit annual status reports to include enrollment, withdrawals, reason for withdrawal, adverse events, and complaints and how they were handled. Since the substudy involves minimal risk, the PI will be responsible for safety monitoring and reporting to the Ethics Committees of UCT and the Seattle Children's IRB.

4. Importance of knowledge to be gained. The knowledge gained by this project has the potential to lead to interventions to mitigate immune activation and inflammation in infants who are HIV-exposed.

5. Sex as a Biological variable. We do not anticipate that infant sex will have an effect on the immunoglobulin-microbe binding profile in the infant gut nor inflammation. Infant sex may influence neonatal immunity, but this variable will be considered in analysis.