EVALUATION OF ANTIHELMINTH TREATMENT AND AN ALOE ENRICHED WHEY PROTEIN DRINK ON MEASURES OF HIV ENTEROPATHY AND IMMUNE ACTIVATION IN HIV+ CHILDREN IN ADDIS ABABA

By

Mary C. Vander Wal

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ABSTRACT

EVALUATION OF ANTIHELMINTH TREATMENT AND AN ALOE ENRICHED WHEY PROTEIN DRINK ON MEASURES OF HIV ENTEROPATHY AND IMMUNE ACTIVATION IN HIV+ CHILDREN IN ADDIS ABABA, ETHIOPIA

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We evaluated microbial translocation, immune activation and recovery in a cohort of HIV+ children in Addis Ababa, Ethiopia pre and post supplementation with an aloe enriched whey protein drink. One hundred and twenty HIV+ children between the ages of four and eleven were enrolled into this twelve-week randomized and blinded clinical trial. Intestinal mucosal barrier integrity was assessed using intestinal fatty acid binding protein (I-FABP) and soluble CD14 (sCD14). Flow cytometry was used to identify T-cell subsets and quantify markers of immune activation and cellular proliferation. Other outcome measures included anthropometric measurements (weights, heights, mid upper arm circumference (MUAC), CD4 counts, viral loads, liver panels and creatinine levels. Results – Gains in height and weight were noted in both the intervention and iso-caloric control groups but did not reach significance in between group analysis. Both groups noted significant decreases in levels of sCD14 ($p = 0.0009$ in control vs 0.0001 in Aloe/Whey group) and significant increases in proliferating naïve CD4 T-cells ($p = 0.003$ in control vs 0.007 in the Aloe/Whey group). The interventions were well tolerated, and no significant adverse events reported.

I dedicate this dissertation, the degree bestowed as a result and all subsequent use of the skills and knowledge that now accompany me in my life journey to my LORD and Savior Jesus Christ. He has called me out to this task and has walked with me the entire course of this lengthy journey whispering words of love and encouragement to keep to the path, look forward and TRUST in his leading.

To God be the glory!

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KEY TO ABBREVIATIONS

HIV- Human immunodeficiency virus

GALT- Gut-associated lymphoid tissue

CD4- Cluster of differentiation 4

CD45RA- Cluster of differentiation RA type isoform

CD62L- Cluster of differentiation L selectin

Ki-67-Monoclonal antibody Ki-67

CCR5- C-C chemokine receptor 5

CXCR4- C-X-C chemokine receptor 4

LPS- Lipopolysaccharide

TAC- Total antioxidant capacity

I-FABP- Intestinal fatty acid binding protein

sCD14- Soluble cluster of differentiation a4

ART-Antiretroviral therapy

IL1β- interleukin 1 beta

IL6-Interleukin 6

TNFα-Tumor necrosis factor alpha

AIDS- Acquired immunodeficiency syndrome

PBRM- Polysaccharide biological response modifiers

PRR- Pattern recognition receptors

MR-Mannose receptor

CRF- Case report form

INTRODUCTION

Human immunodeficiency virus (HIV) causes a complex disease with multiple ramifications within the body. Since the beginning of the HIV epidemic in the 1980s great strides have been made in understanding this disease. Drug development that targets specific mechanisms of viral replication and combination regimens that provide greater effectiveness and less toxicity have become common place; however, there is still much to learn about managing the devastating effects of this disease. Immune activation is a hallmark of HIV disease and has been linked to several causes including the viral infection itself, co-infections with other pathogens, medications used to treat the viral infection, high risk life styles and microbial translocation (Hunt et al., 2014; Paiardini & Müller-Trutwin, 2013). This last cause, microbial translocation, is a newer focus of HIV research in both animal and human models and is felt to be a major contributor to the chronic immune activation with resultant systemic inflammation and the development of non-HIV co-morbidities (Brenchley, et al., 2006; Deeks, 2011; Klatt, Funderburg, & Brenchley, 2013; Sandler & Douek, 2012).

It is now known that the Gut-Associated Lymphoid Tissue (GALT), as well as being the primary site of initial infection and viral replication, continues to be a site of ongoing pathology (Brenchley & Douek, 2008; Dandekar, 2007). Compromised mucosal barrier function, which develops early in the disease, allows translocation of bacterial and viral components into the general circulation, initiating a state of chronic immune activation which impacts disease progression, and contributes to the development of multiple co-morbidities (Appay & Sauce, 2008; Brenchley et al., 2006; Klatt et al., 2013). Correlations between measures of microbial translocation and elevated immune

activation markers have been documented repeatedly in HIV+ adults and are beginning to be documented in HIV+ children (Brenchley et al., 2006; Giorgi et al., 1999; Marchetti, Tincati, & Silvestri, 2013; Stephensen, Marquis, Douglas, & Wilson, 2005; Wallet et al., 2010).

Over the 30+ years since the beginning of the AIDS epidemic, survival rates have improved dramatically, and life expectancy has been extended by decades. In spite of this, life expectancy for those infected with HIV is still cut short by several years with mortality often now being attributed to non-HIV co-morbidities rather than to the viral infection itself (Bhatia, Ryscavage, & Taiwo, 2012; Deeks, 2011; Franceschi & Campisi, 2014; Kuller et al., 2008; Smith, Boer, Brul, Budovskaya, & Spek, 2013). These comorbidities include cardiovascular disease, diabetes, renal disease, cancers, neurocognitive decline and osteoporosis (Appay & Sauce, 2008; Duprez et al., 2012; Hunt et al., 2014; Sandler & Douek, 2012). Adult HIV cohorts, even when well-controlled on antiretroviral therapy (ART) and with non-detectable viral loads, have higher markers of immune activation, oxidative stress, and systemic inflammation than healthy controls and develop age-related co-morbidities at earlier ages (Bhatia et al., 2012; Deeks, 2011; Hunt et al., 2014; Smith et al., 2013). The “normal” aging process, though not fully understood, is similar in that it involves immune activation, oxidative stress and chronic inflammation which has led to the term “inflamaging” (Deeks, 2011; Franceschi & Campisi, 2014).

There are an estimated 2.1 million children living with HIV worldwide (WHO, 2016). Most children under the age of 15 were infected vertically from their HIV+ mother either in-utero, during delivery or as a result of breast feeding (Abel, 2009; Tobin &

Aldrovandi, 2013). The vast majority of HIV+ children reside in resource-poor countries where prenatal care, testing and treatment is not as consistent as in developed countries. Children in resource-poor countries often face additional challenges such as malnutrition and endemic parasites which negatively contribute to the clinical picture of this chronic disease (Embree, 2005).

Little is known about levels of chronic immune activation and inflammation in HIV+ children or what the magnitude of impact this will have over time; however a few beginning trials have shown deleterious effects (DiMeglio et al., 2013; Dimock et al., 2011; Lainka, Oezbek, Falck, Ndagijimana, & Niehues, 2002; Miller et al., 2012a). The development of co-morbid disease in younger HIV populations has significant negative impacts on personal health and well-being and will increase the financial strain on already fragile healthcare systems in the developing world where most of those impacted reside.

Current therapies to treat HIV are targeted at eradicating viral replication and as such are not designed to address this pathology in the gut that has significant impact on disease progression and health outcomes. For poorly understood reasons, ART is not effective at eradicating viral reserves in the GALT and though reduced, the pathology in the gut continues (Brenchley & Douek, 2008; Dandekar, 2007; Klatt et al., 2013). Research exploring the presence and impacts of chronic immune activation, oxidative stress, and systemic inflammation in pediatric HIV cohorts is confirming the impacts in younger cohorts. Innovative therapies with the potential to delay the development of non-HIV morbidities are urgently needed.

Various plant, herbal and nutritional interventions are being evaluated for their

anti-inflammatory properties. Both amino acid blends and aloe polysaccharides have been demonstrated to support tissue healing and attenuate release of pro-inflammatory cytokines and free radical production in animal and human models of colitis. (Budai, Varga, Milesz, Tőzsér, & Benkő, 2013; Davis, 1994; Faure et al., 2006; Im et al., 2010; Koetzner, Grover, Boulet, & Jacoby, 2010; Petyaev, Dovgalevsky, Klochkov, Chalyk, & Kyle, 2012; Sprong, Schonewille, & van der Meer, 2010). Either or both nutritional supplements may be of benefit for individuals with HIV, however neither has been evaluated specifically on measures of HIV enteropathy.

The central aim of this research was to quantify the impacts of two nutritional supplements; whey protein (WP) and an Aloe Vera powder (AVP), on measures of microbial translocation, immune activation and clinical outcomes in HIV+ children living in Addis Ababa, Ethiopia.

Specific Aim 1: To evaluate intestinal mucosal barrier function in this population by assessing measures of microbial translocation by quantifying levels of intestinal fatty-acid binding protein (I-FABP) and soluble cluster of differentiation 14 (sCD14) pre and post supplementation.

Hypothesis: The daily ingestion of an aloe enriched whey protein drink will improve mucosal barrier function with a resultant decrease in plasma measurements of I-FABP and sCD14.

Specific Aim 2: To assess levels of immune activation (CD4/CD8 ratio, HLA-DR+ CD38+CD8+) before and after treatment for helminths and supplementation with aloe-enriched whey protein.

Hypothesis 2a: One-time treatment with antihelminths will decrease levels of immune

activation from baseline measures.

Hypothesis 2b: Treatment with antihelminths combined with daily supplementation with an aloe-enriched whey protein drink will result in greater decreases in levels of immune activation than treatment with antihelminths in combination with a daily isocaloric drink.

Specific aim 3: To evaluate the functional impact of helminthic treatment alone or in combination with nutritional support on anthropometric measures and immune reconstitution; specifically, total CD4 counts, as well as CD4 T subsets indicative of ongoing immune reconstitution, namely, recent thymic emigrants (CD45RA+CD62L+CD31+) and cells generated by homeostatic proliferation (CD45RA+CD62L+Ki67+).

Hypothesis 3a: Antihelminth treatment in combination with a daily aloe-enriched whey protein drink will result in greater gains in 1 or more measures of anthropometric measurements than antihelminth treatment in combination with a daily isocaloric drink.

Hypothesis 3b: Antihelminth treatment in combination with a daily aloe-enriched whey protein drink will result in greater gains in 1 or more measures of immune reconstitution than antihelminth treatment in combination with a daily isocaloric drink.

REFERENCES

REFERENCES

Abel, K. (2009). The rhesus macaque pediatric SIV infection model - a valuable tool in understanding infant HIV-1 pathogenesis and for designing pediatric HIV-1 prevention strategies. *Current HIV Research*, *7*(1), 2–11.

Appay, V., & Sauce, D. (2008). Immune activation and inflammation in HIV-1 infection: causes and consequences. *The Journal of Pathology*, *214*(2), 231–241. https://doi.org/10.1002/path.2276

Bhatia, R., Ryscavage, P., & Taiwo, B. (2012). Accelerated aging and human immunodeficiency virus infection: emerging challenges of growing older in the era of successful antiretroviral therapy. *Journal of Neurovirology*, *18*(4), 247–255. https://doi.org/10.1007/s13365-011-0073-y

Brenchley, J. M., & Douek, D. C. (2008). HIV infection and the gastrointestinal immune system. *Mucosal Immunology*, *1*(1), 23–30. https://doi.org/10.1038/mi.2007.1

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., … Douek, D. C. (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature Medicine*, *12*(12), 1365–1371. https://doi.org/10.1038/nm1511

Budai, M. M., Varga, A., Milesz, S., Tőzsér, J., & Benkő, S. (2013). Aloe vera downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages. *Molecular Immunology*, *56*(4), 471–479. https://doi.org/10.1016/j.molimm.2013.05.005

Dandekar, S. (2007). Pathogenesis of HIV in the gastrointestinal tract. *Current HIV/AIDS Reports*, *4*(1), 10–15. https://www.ncbi.nlm.nih.gov/pubmed/17338855

Davis, R. H., Donato, J. J., Hartman, G. M., & Haas, R. C. (1994). Anti-inflammatory and wound healing activity of a growth substance in Aloe vera. *Journal of the American Podiatric Medical Association*, *84*(2), 77–81. https://doi.org/10.7547/87507315-84-2-77

Deeks, S. G. (2011). HIV Infection, Inflammation, Immunosenescence, and Aging. *Annual Review of Medicine*, *62*(1), 141–155. https://doi.org/10.1146/annurev-med-042909-093756

DiMeglio, L. A., Wang, J., Siberry, G. K., Miller, T. L., Geffner, M. E., Hazra, R., … Jacobson, D. L. (2013). Bone mineral density in children and adolescents with perinatal HIV infection: *AIDS*, *27*(2), 211–220. https://doi.org/10.1097/QAD.0b013e32835a9b80

Dimock, D., Thomas, V., Cushing, A., Purdy, J. B., Worrell, C., Kopp, J. B., … Hadigan, C. (2011). Longitudinal assessment of metabolic abnormalities in adolescents and young adults with HIV-infection acquired perinatally or in early childhood. *Metabolism*, *60*(6), 874–880. https://doi.org/10.1016/j.metabol.2010.08.007

Duprez, D. A., Neuhaus, J., Kuller, L. H., Tracy, R., Belloso, W., De Wit, S., … Neaton, J for the INSIGHT SMART Study Group. (2012). Inflammation, Coagulation and Cardiovascular Disease in HIV-Infected Individuals. *PLoS ONE*, *7*(9), e44454. https://doi.org/10.1371/journal.pone.0044454

Embree, J. (2005), The impact of HIV/AIDS on children in developing countries. *Paediatrics & child health,* 10(5), 261-263. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2722540/

Faure, M., Mettraux, C., Moennoz, D., Godin, J.-P., Vuichoud, J., Rochat, F., … Corthésy-Theulaz, I. (2006). Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *The Journal of Nutrition*, *136*(6), 1558–1564.

Franceschi, C., & Campisi, J. (2014). Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *69*(Suppl 1), S4–S9. https://doi.org/10.1093/gerona/glu057

Giorgi, J. V., Hultin, L. E., McKeating, J. A., Johnson, T. D., Owens, B., Jacobson, L. P., … Detels, R. (1999). Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *The Journal of Infectious Diseases*, *179*(4), 859–870. https://doi.org/10.1086/314660

Hunt, P. W., Sinclair, E., Rodriguez, B., Shive, C., Clagett, B., Funderburg, N., … Lederman, M. M. (2014). Gut Epithelial Barrier Dysfunction and Innate Immune Activation Predict Mortality in Treated HIV Infection. *Journal of Infectious Diseases*, *210*(8), 1228–1238. https://doi.org/10.1093/infdis/jiu238

Im, S.-A., Lee, Y.-R., Lee, Y.-H., Lee, M.-K., Park, Y. I., Lee, S., … Lee, C.-K. (2010). In vivo evidence of the immunomodulatory activity of orally administered Aloe vera gel. *Archives of Pharmacal Research*, *33*(3), 451–456. https://doi.org/10.1007/s12272-010-0315-1

Klatt, N. R., Funderburg, N. T., & Brenchley, J. M. (2013). Microbial translocation, immune activation, and HIV disease. *Trends in Microbiology*, *21*(1), 6–13. https://doi.org/10.1016/j.tim.2012.09.001

Koetzner, L., Grover, G., Boulet, J., & Jacoby, H. I. (2010). Plant-Derived Polysaccharide Supplements Inhibit Dextran Sulfate Sodium-Induced Colitis in

the Rat. *Digestive Diseases and Sciences*, *55*(5), 1278–1285. https://doi.org/10.1007/s10620-009-0848-7

Kuller, L. H., Tracy, R., Belloso, W., Wit, S. D., Drummond, F., Lane, H. C., … for the INSIGHT SMART Study Group. (2008). Inflammatory and Coagulation Biomarkers and Mortality in Patients with HIV Infection. *PLoS Medicine*, *5*(10), e203. https://doi.org/10.1371/journal.pmed.0050203

Lainka, E., Oezbek, S., Falck, M., Ndagijimana, J., & Niehues, T. (2002). Marked dyslipidemia in human immunodeficiency virus-infected children on protease inhibitor-containing antiretroviral therapy. *Pediatrics*, *110*(5), e56.

Marchetti, G., Tincati, C., & Silvestri, G. (2013). Microbial Translocation in the Pathogenesis of HIV Infection and AIDS. *Clinical Microbiology Reviews*, *26*(1), 2–18. https://doi.org/10.1128/CMR.00050-12

Miller, T., Borkowsky, W., DiMeglio, L., Dooley, L., Geffner, M., Hazra, R., … Pediatric HIV/AIDS Cohort Study (PHACS). (2012a). Metabolic abnormalities and viral replication are associated with biomarkers of vascular dysfunction in HIV-infected children: Vascular dysfunction in paediatric HIV infection. *HIV Medicine*, *13*(5), 264–275. https://doi.org/10.1111/j.1468-1293.2011.00970.x

Paiardini, M., & Müller-Trutwin, M. (2013). HIV-associated chronic immune activation. *Immunological Reviews*, *254*(1), 78–101. https://doi.org/10.1111/imr.12079

Petyaev, I. M., Dovgalevsky, P. Y., Klochkov, V. A., Chalyk, N. E., & Kyle, N. (2012). Whey Protein Lycosome Formulation Improves Vascular Functions and Plasma Lipids with Reduction of Markers of Inflammation and Oxidative Stress in Prehypertension. *The Scientific World Journal*, *2012*, 1–7. https://doi.org/10.1100/2012/269476

Sandler, N. G., & Douek, D. C. (2012). Microbial translocation in HIV infection: causes, consequences and treatment opportunities. *Nature Reviews Microbiology*, *10*(9), 655–666. https://doi.org/10.1038/nrmicro2848

Smith, R. L., Boer, R. de, Brul, S., Budovskaya, Y., & Spek, H. van der. (2013). Premature and accelerated aging: HIV or HAART? *Frontiers in Genetics*, *3*. https://doi.org/10.3389/fgene.2012.00328

Sprong, R. C., Schonewille, A. J., & van der Meer, R. (2010). Dietary cheese whey protein protects rats against mild dextran sulfate sodium–induced colitis: Role of mucin and microbiota. *Journal of Dairy Science*, *93*(4), 1364–1371. https://doi.org/10.3168/jds.2009-2397

Tobin, N. H., & Aldrovandi, G. M. (2013). Immunology of pediatric HIV infection. *Immunological Reviews*, *254*(1), 143–169. https://doi.org/10.1111/imr.12074

Wallet, M. A., Rodriguez, C. A., Yin, L., Saporta, S., Chinratanapisit, S., Hou, W., … Goodenow, M. M. (2010). Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T-cell activation following therapy: *AIDS*, *24*(9), 1281–1290. https://doi.org/10.1097/QAD.0b013e328339e228

WHO | Data and statistics. (2016). Retrieved December 7, 2016, from http://www.who.int/hiv/data/epi_core_2016.png?ua=1

CHAPTER 1

Background and Literature Review

HIV and Gut Mucosa

The initiation of an infection with HIV requires binding of the virus to cells expressing cluster of differentiation 4 (CD4) markers on their outer membranes as well as attachment to one of two co-receptors. The predominant co-receptor used to gain entry is C-C chemokine receptor 5 (CCR5) and to a lesser extent C-X-C chemokine receptor 4 (CXCR4), CD4+ T cells express CCR5 upon activation whereas naïve T cells express little to no CCR5 (Fauci, 2007). Activated T cells are therefore the predominant target for viral attachment. The gut associated lymphoid tissue (GALT) is home to the largest populations of activated CD4+CCR5+ T cells in the body where they function as memory cells and provide immune surveillance for the massive surface area in the gut (Fauci, 2007).

These memory CD4+ T cell populations in the GALT undergo massive depletion within the first few days of infection even before drops in peripheral populations of CD4+ cells are detectable (Brenchley & Douek, 2008; Klatt, Funderburg & Brenchley, 2013; Sandler & Douek, 2012; Veazey, R & Lackner, A, 2003). Their numbers remain compromised throughout the disease in spite of good treatment response to ART with non-detectable levels of virus in peripheral blood (Brenchley et al., 2006; Hunt et al., 2014, 2014; Paiardini & Müller-Trutwin, 2013). It is now recognized that the GALT is the predominant site of viral attachment and initiation of primary HIV infection in both adults and children regardless of route of infection (Brenchley et al., 2006; Castello-Branco, Lewis, Ortigão-de-Sampaio, & Griffin, 1996; Dandekar, 2007; Sandler & Douek, 2012).

At the same time that CD4+ T cells in the gut mucosa are being depleted an intense immune response is being mounted. Numbers of activated CD8+ T cells increase significantly in a compensatory attempt to eradicate the infection. These cytotoxic cells in the GALT are partially responsible for the rapid loss of CD4 cells and also for the initiation of an inflammatory response in the gut mucosal tissue. Locally released cytokines, as well as apoptotic mechanisms, result in the loss of intestinal epithelial cells and damage to their tight junctions (Brenchley & Douek, 2008). Compromise of the gut mucosal barrier allows bacterial and viral particles that are usually well maintained within the lumen of the gut to traverse the epithelial layer.

Lipopolysaccharide (LPS), also known as endotoxin, is a breakdown product from the cell wall of gram-negative bacteria, and is a very potent stimulator of immune cells, however macrophage that have homed to the GI tract develop a tolerogenic phenotype and so do not become activated on exposure with gut microbiota or express/secrete inflammatory cytokines. Numerous macrophage are stationed within the lamina propria of the submucosal tissues of the GI tract and these macrophage generally are able to bind, phagocytize and digest bacterial products that manage to leak through tight junctions, however, compromised macrophage activity associated with HIV allows translocation of these particles into the peripheral circulation (Brenchley et al., 2006; Klatt, Funderburg & Brenchley, 2013). When LPS from the intestinal lumen traverses into the general circulation it comes in contact with circulating monocytes and other innate immune cells which become activated and secrete numerous inflammatory cytokines and chemokines including IL-1β, IL-6 and TNF-α. Monocytes also secrete soluble cluster of differentiation 14 (sCD14), a co-receptor for LPS, which, in complex

with lipopolysaccharide binding protein (LBP), binds and neutralizes LPS.

This pathology in the gut, with or without the presence of non-infectious diarrhea, has been termed HIV enteropathy and is very similar to other inflammatory bowel diseases with varying degrees of severity producing symptoms and conditions of diarrhea, mucosal inflammation, malabsorption and wasting (Brenchley & Douek, 2008; Sandler & Douek, 2012; Zeitz et al., 1998). HIV enteropathy has been shown in primates to result in villous atrophy and crypt cell hyper proliferation in the mucosal lining leading to malabsorption (Kewenig et al., 1999; Zeitz et al., 1998).

HIV enteropathy was a term coined early into the HIV epidemic however the cause of this enteropathy was not well understood. Researchers in the last decade have brought to light the ongoing pathology in the GALT of HIV+ individuals and the profound impacts this pathology has on disease progression and the subsequent development of comorbidities.

HIV Enteropathy and Immune Activation

A major finding in HIV research was the discovery of LPS in the serum of HIV+ patients. The spilling of LPS into the general circulation is indicative of compromised tight epithelial junctions. In newborns this is a normal finding which does not cause immune activation and which clears over the first few months; however, in older infants, children and adults this is associated with inflammation of the gut mucosa, following intestinal surgery or as a sequelea of graft vs host disease (Pilakka-Kanthikeel et al., 2012; Tobin & Aldrovandi, 2013). The presence of LPS in the general circulation causes circulating monocytes and macrophages to be activated and release soluble cluster of differentiation 14 (sCD14). The CD14 molecule, in complex with lipopolysaccharide

binding protein (LBP), binds LPS and neutralizes it. Therefore, sCD14 and LBP have become recognized markers of microbial translocation. Another consequence of LPS stimulation, as well as several other less understood mechanisms, is the induction of pro-inflammatory molecules including IL-1β, IL-2, IL-6, IL-8, IFNβ, IFN-γ, and TNFα. Chronic secretion of these inflammatory cytokines results in a systemic inflammatory state, with elevations of markers such as C-reactive protein (CRP), neopterin, cystatin C and D-dimers. Chronic inflammation has been correlated repeatedly with the development of comorbid diseases and aging in various populations beyond HIV (Blanco, J, 2012; Brenchley et al., 2006; Deeks, 2011; Klatt, Funderburg & Brenchley, 2013). In addition, chronic T cell activation provides an ongoing source of CCR5+ T cells for viral attachment, initiating a vicious cycle of viremia, gut mucosal damage, immune activation and systemic inflammation. This results in continuous T cell attrition in varying degrees throughout the course of the disease (Appay & Sauce, 2008; Wallet et al., 2010)

Giorgi et al. (1999) noted that shortened survival rates of individuals with HIV were more closely correlated with immune activation markers than with viral loads; however, the causes of the chronic immune activation seen in HIV were still elusive. Brenchley et al. (2006) conducted one of the first explorations into the connections between gut pathology, immune activation and HIV progression. In their very thorough team approach they analyzed blood samples from 205 HIV+ adults at different stages of disease and treatment from different treatment centers and compared them with 45 HIV- adults. In addition, they infected 11 rhesus macaques with simian immunodeficiency virus (SIV) and after 100 days collected samples from these as well

as from 12 SIV infected sooty mangabeys and 11 uninfected sooty mangabeys. Rhesus monkeys are often used in HIV research due to the similarities to humans they display in responses to the SIV virus. Sooty mangabeys as well as African green monkeys, on the other hand, are both natural SIV hosts. As such, when infected, these species develop high rates of viremia but do not develop gut pathology or immune activation and do not progress to acquired immune deficiency (AIDS). In all of these cohorts, measures of LPS, sCD14, LPS binding protein (LBP), endotoxin-core antibodies (EndoCab), interferon gamma (INFγ), as well as viral loads were monitored and compared. LPS levels in all of the human HIV cohorts were significantly higher ($p < 0.0001$) than healthy controls. Decreased levels of LPS were documented in those who had been on maintained on ART, however their levels of LPS were still significantly higher than controls ($p = 0.0026$) and levels of sCD14 were not significantly reduced even after 48 weeks of treatment ($p = 0.87$). Measures of both sCD14 and LBP were significantly higher in infected human cohorts compared to healthy controls ($p < 0.0001$ and $p = 0.0099$ respectively) and elevated LPS was significantly correlated with sCD14 ($p = 0.001$) providing support for a direct stimulatory effect. There was no significant correlation noted between sCD14, LPS or CD4 counts with plasma viral load ($p = 0.5$, $p = 0.226$ and $p = 0.492$ respectively). Measures of plasma EndoCab, which also binds and neutralizes LPS, were elevated in the acute/early disease cohort compared to controls ($p < 0.0001$). However, in individuals with chronic HIV, EndoCab levels were significantly lower than controls or those in the acute/early cohort suggesting that as the infection becomes chronic, levels of EndoCab are no longer able/available to neutralize LPS. A documented inverse correlation between EndoCab titers and LPS ($p = 0.0005$)

led to this conclusion by the authors. Ten of the 11 rhesus macaques demonstrated elevated levels of LPS compared to baseline. To help to illuminate the source of the LPS, two of the infected treatment naive rhesus macaques were given a triple antibiotic course to “sterilize” the bowel. After 1 week of the antibiotic regimen plasma LPS levels dropped in tandem with decreases in gram negative bacteria in stool samples, supporting the assumption that plasma LPS was being deposited into the general circulation through leaking intestinal mucosal ports. Of interest and great significance was the evaluations done in sooty mangabeys. Post infection viral loads climbed as expected, but even in the presence of high-level viremia these hosts did not develop levels of LPS or immune activation markers significantly higher than uninfected controls. SIV infected sooty mangabeys experienced rapid depletion of gut mucosal CD4 T cells but not to the extent that their cousins the rhesus macaques did, and they did not display the damage to mucosal tissues or display microbial translocation as is noted in rhesus macaques or humans. This provided added support that chronic immune activation in HIV populations is in large part caused by the ongoing pathology in the gut mucosa.

Other researchers repeating similar trial designs have reaffirmed correlations between microbial translocation, chronic immune activation and disease progression. In addition to LPS and sCD14, other markers of microbial translocation have been utilized including EndoCab, 16S rDNA (an rRNA subunit common to most bacteria), and intestinal fatty acid binding protein (I-FABP or FABP2), an intracellular protein specific to enterocytes that is released with cell damage). While some trials have not noted direct correlations between LPS levels and immune activation, nearly all have made links

using other markers of microbial translocation and have concluded that HIV enteropathy is a major contributor to chronic immune activation (Asmuth et al., 2013; Brenchley et al., 2006; Deeks, 2011; Duprez et al., 2012; Hunt et al., 2014; Kuller et al., 2008; Neuhaus et al., 2010; Pilakka-Kanthikeel et al., 2012; Sandler et al., 2011; Wallet et al., 2010). The lack of consistent correlations with LPS may be due to stage of disease coupled with small sample sizes as well as the difficulty in capturing accurate levels of LPS due to inhibitory protein binding and the need to perform these assays in a pyrogen-free environment. Levels of LPS and other markers of microbial translocation do decrease with the initiation of ART; however, in almost all investigations, levels remain elevated above controls even after several years of treatment and non-detectable levels of virus (Canipe et al., 2014; French, King, Tschampa, da Silva, & Landay, 2009; Hunt et al., 2014; Sandler et al., 2011).

Immune activation in HIV populations has been attributed to several mechanisms; some better understood than others and some likely contributing more to the process. The key identified contributors in HIV populations are: 1) Pathogens- mainly the HIV virus itself but also other co-infections such as cytomegalovirus (CMV), tuberculosis (TB) or hepatitis B or C, that set off antigenic responses resulting in immune activation; 2) Lifestyle choices prevalent in HIV populations- including high risk sexual behavior, drug use and abuse, as well as high rates of alcohol use have been linked to chronic immune activation.; 3) Antiretroviral drugs-these therapies themselves create mitochondrial stress, increasing free radical burden and increasing immune activation; 4) Endemic intestinal parasites- especially in pediatric populations in developing countries are a contributing cause of immune activation; 5) HIV enteropathy.

As discussed, it is now well acknowledged that a very significant cause of sustained immune activation in HIV populations is microbial translocation from the gut.

Immune Activation and Chronic Inflammation

Immune activation is initiated by the transformation of cells to an activated phenotype and involves a variety of cell types including monocytes, macrophages, dendritic cells and lymphocytes. When activated these cells secrete pro-inflammatory cytokines, cytotoxic granules, or attach to receptors of target cells and initiate apoptosis, all for the purpose of eliminating an antigenic threat. Cytokines have short half-lives, and the secretion of inflammatory cytokines is generally a short-term event intended to recruit members of the innate and adaptive immune system and to initiate tissue repair (Germolec, Frawley, & Evans, 2010). Immune activation is an appropriate and desired response to invasion of pathogens or trauma; however, the continual exposure of tissues and vessels to these inflammatory molecules leads to the development of chronic inflammation with tissue damage and fibrosis as well as immune senescence and dysfunction (Appay & Sauce, 2008; Deeks, 2011; Germolec et al., 2010). It is now known that virtually all age-related diseases are closely tied to inflammation, hence the coined term “inflammaging”. Several markers of inflammation, including IL-1β, IL-6, TNFα, c-reactive protein (CRP), neopterin, and cystatin C, have been utilized in establishing correlations with comorbidities such as cardiovascular disease, renal disease, osteoporosis, cognitive decline and the development of various cancers (Appay & Sauce, 2008; Deeks, 2011; Franceschi & Campisi, 2014; Germolec et al., 2010).

Years of intense research has led to improved multidrug regimens for HIV that

have increased life expectancies by decades (Klatt, Funderburg & Brenchley, 2013; Marchetti et al., 2013). This extension of life has also allowed for the realization and exploration of the premature development of co-morbid disease and the increased incidence of non-HIV associated mortality in this population. Numerous trials have been conducted in adult HIV populations confirming that increased expression of inflammatory markers leads to the establishment of co-morbid disease (Blodget et al., 2012; Burdo et al., 2011; Canipe et al., 2014; Longenecker et al., 2013; Sandler et al., 2011).

The Strategies for Management of Anti-Retroviral Therapy (SMART)(2006), although not designed for this purpose, gathered large amounts of data that allowed for the substantiation of links between immune activation, inflammation and the development of comorbidities and mortality. The SMART study was designed to evaluate intermittent versus continuous daily administration of ART to see if there would be an impact on viral loads and immune recovery with a less rigid dosing regimen. The motivation for conducting this study was the number of adverse side effects and events connected to ART use and the financial burden of everyday use of these expensive multidrug regimens. Between 2002 and 2006, 5,472 HIV+ individuals ages 14 years and older, with CD4 counts above 350, were enrolled from 33 countries and randomized to either a continuous daily ART (viral suppression) group or an intermittent ART (drug conservation) group with varying dosing cycles built into this group. The study had planned to enroll 6,000 participants, each for six consecutive years; however, it was terminated early in 2006 due to the preponderance of significant adverse cardiovascular, renal, and hepatic events and deaths in the drug conservation group as

compared to the viral suppression group. One hundred twenty participants from the drug conservation group vs 47 in the viral suppression group developed opportunistic diseases or died. The major cause of death was cancers. Also, 65 participants from the drug conservation group developed one or more serious cardiac, renal or hepatic events versus 39 in the viral suppression group (hazard ratio, 1.7; 95% CI, 1.1 to 2.5; $p = 0.009$). The study authors clearly demonstrated that continuous viral suppression therapy was advantageous; however, an interesting and unexpected discovery was that the majority of the deaths and significant non-HIV adverse events, primarily cardiovascular, occurred in the drug conservation group. The researchers had anticipated that the frequency of non-HIV adverse events were attributed to drug toxicities and envisioned lower rates in the drug conservation group; however, this was not the case (SMART) Study Group et al 2006).

One very positive outcome of this trial was to ignite further research focused on the connections between immune activation and inflammation in HIV disease and the development of comorbid diseases (Hearps, Martin, Rajasuriar, & Crowe, 2014). Stored plasma samples from this multicountry, multiethnic trial have been used to compare markers of immune activation and inflammation with samples from other large population-based multiethnic trials such as the Multi-Ethnic Study of Atherosclerosis (MESA) and the Coronary Artery Development in Young Adults (CARDIA). These comparisons have consistently reported correlations between elevated markers of immune activation and systemic inflammation and increased frequencies of co-morbid disease and mortality in HIV populations (Duprez et al., 2012; Kuller et al., 2008; Neuhaus et al., 2010).

Multiple trials in adults have now linked chronic immune activation with the development of comorbid disease in HIV adults. While all comorbid disease development has implications for the pediatric HIV population, one area of significant concern is the impact on bone health. A large meta-analysis showed that two thirds of HIV+ adults have low bone mineral density (Brown & Qaqish, 2006) and risks of fracture in an adult cohort were reported to be 30-70% higher than controls (Puthanakit et al., 2012).

Not all trials have reported links between microbial translocation and comorbidities. One contradicting trial, conducted by Longenecker et al. (2013), reported that monocyte activation markers (CD14, CD16 and tissue factor (TF)), and markers of microbial translocation, did not correlate with developing signs of carotid artery disease. They unfortunately did not evaluate LPS levels. They did find strong correlations between CD8 T cell activation and markers of inflammation with common carotid artery (CCA) intima-media thickness (IMT) and carotid plaque formation (Longenecker et al., 2013). In this study of 60 individuals, 78% were men, 58% were smokers, 35% had a family history of heart attack and 45% were on protease inhibitors which have been linked to endothelial dysfunction. While this trial did not note a direct correlation between microbial translocation and the development of cardiovascular disease (CVD), it did show the presence of immune activation and inflammation in this cohort and highlighted the complexities of seeking correlations when multiple factors may influence these associations.

HIV and Oxidative Stress

As noted, there are several contributors to the development of comorbidities in

HIV. One area of focus is the chronic state of oxidative stress that joins the picture when chronic immune activation and inflammation are present. Oxidative stress is a state of imbalance when the body’s antioxidant reserves are not able to keep up with the production of harmful ROS. Enzymatic (superoxide dismutase, glutathione peroxidase, catalase, etc.) as well as non-enzymatic (bioflavonoids, uric acid, tocopherols, carotenoids, bilirubin, etc.) systems within the body help to balance the production of reactive oxygen species (ROS). Oxidative stress is common in all chronic disease states, and with HIV is brought on not only by the viral infection itself but also by the ART used to treat it (Coaccioli et al., 2010; Conway & Tossonian, 2014; Gil et al., 2003; Smith et al., 2013). Oxidative stress is linked to immune activation and inflammation and its presence has a negative impact on disease progression and immune recovery (Coaccioli et al., 2010; Suresh, Annam, Pratibha, & Prasad, 2009). Since free radical research is a fairly new area of exploration, Suresh conducted a study to evaluate appropriate markers as well as assess impacts of HIV on oxidative status. Fifty HIV+ treatment-naive adults (31men and 19 women) were compared against 50 matched controls for measures of total antioxidant capacity, vitamin E and vitamin C levels and superoxide dismutase as well as quantifications of lipid peroxidation by measurement of malondialdehyde (MDA). The HIV+ participants were further categorized as symptomatic (CD4 <500cell/ml) versus asymptomatic (CD4 >500cells/ml). Participants were excluded with known opportunistic infections, histories of smoking, co-morbid diseases, supplemental vitamin use, hyperlipidemia, chronic diarrhea or vomiting, or evidence of GI bleeding. MDA levels were significantly increased across the HIV+ group ($p \leq 0.01$) which is indicative of lipid peroxidation. Levels of all individual antioxidants as

well as measures of total antioxidant capacity (TAC) were also significantly lower in the HIV+ groups ($p < 0.01$) and were significantly lower in the symptomatic group compared to the asymptomatic group suggesting severe and progressive oxidative stress in these groups before the initiation of ART which has been documented to lead to further oxidative stress (Sharma, 2014).

In summary, HIV infection leads to early and severe loss of CD4 T cells in the GALT that triggers an immune response and leads to loss of tight junctions between enterocytes and the spillage of microbial particles into the general circulation. This is an ongoing process leading to chronic immune activation, setting the stage for systemic inflammation and the development in of non-HIV comorbidities in this population several years earlier than in the general population. HIV research conducted with adult populations has consistently reported links between immune activation, chronic immune activation, oxidative stress and the early development of age-related comorbidities. It is very difficult, however, to isolate contributors to immune activation and development of comorbidities in adult populations even when measures have been put into place to control for confounding variables such as diet, smoking, drug and alcohol use and abuse or other lifestyle contributors. Also, these well-documented impacts have involved adults with HIV who contracted the infection as adults, but what is the picture and future of children who contract the infection in utero or during the antenatal period? It is not possible to make extrapolations from this data to pediatric HIV populations. The clinical impacts of a chronic low-grade inflammatory state in children with HIV may not be fully appreciated for many years; however, elevation of inflammatory markers in children are starting to be documented and this chronic inflammation is likely to increase

the risks of comorbidities seen in HIV+ adult populations but at much earlier ages (DiMeglio et al., 2013; Khaitan et al., 2016; Miller et al., 2012; Pilakka-Kanthikeel et al., 2012; Plaeger-Marshall et al., 1994; Resino, Seoane, Gutiérrez, León, & Muñoz-Fernández, 2006; Roider, Muenchhoff, & Goulder, 2016).

The rest of this paper will be looking at the research in regard to the potential development of co-morbid disease in pediatric HIV populations and the exploration of a nutritional intervention to address this.

Pediatric HIV

It is well-recognized that children are not just little adults. There are many differences in metabolic rates, immune responses, thymic output, as well as growth and tissue healing rates. When we consider pediatric HIV, there are differences in viral initiation and progression as well as immune reconstitution.

Routes for transmission of HIV are different between adults and children with over 95% of children under 15 having contracted the virus in utero, during delivery or over the course of breastfeeding (Sohn & Hazra, 2013). Mother to child transmission (MTCT) was initially assumed to occur with transfer of the virus across placental membranes; however, it is now known that only a small percentage of exposed fetuses contract the virus through this route, most likely due to the fact that the fetal immune environment remains in a quiescent state with cells maintaining a naive phenotype (CCR5-) which do not present the required co-receptor for viral attachment (Tobin & Aldrovandi, 2013). In an attempt to verify transmission routes in humans, Bunders, et al. 2012, performed autopsies on 4 premature and term infants who died in childbirth or shortly after and confirmed a lack of CCR5+CD4 T cells in either the lymph nodes or

cord blood (1% and 0% respectively) in these neonates; however, they noted an abundance of CCR5+ CD4+ T cells in the intestinal mucosa at the time of birth that allow for attachment of virus either through swallowed maternal secretions during delivery or through ingestion of breastmilk.

Factors that dictate the risk of MTCT include the viral load of the mother, the maintenance of the mother on ART, the presence of co-infections in the mother and the gestational age of the infant on exposure. Thanks to heightened surveillance in pregnancy, readily available ART, limited co-infection rates and access to antenatal care; the prevalence of HIV in neonates has become rare in western countries (Abel, 2009; Sohn & Hazra, 2013; Tobin & Aldrovandi, 2013). This is not the case in developing countries where large populations of HIV+ women live and health care resources are limited. It is estimated that over 90% of pediatric HIV, upwards of 2.1 million children, is found in developing countries (Abel, 2009; Tobin & Aldrovandi, 2013).

Simian models of SIV have proved very useful for evaluating the route of initial infection in fetuses and newborns, and it is now known that only 5-10% of fetuses are infected in utero while a larger portion of infections (10-20%) occur antenatally during the passage through the birth canal and 5-10% with transfer of the virus during breast feeding (Tobin & Aldrovandi, 2013). The low rates of HIV found in breast fed infants is surprising as breast feeding allows for repeated exposures daily to extremely large numbers of viral copies (Abel, 2009; Tobin & Aldrovandi, 2013) Breast feeding for HIV women in western countries is discouraged in an effort to lower transmission; however in developing countries, where the risk of mortality from poor sanitation of bottles and formula is high, recommendations support exclusive breast feeding for the first year

(Abel, 2009; Tobin & Aldrovandi, 2013).

Amazingly, only about 20% of infants develop infection by any route; however, infants who contract the virus in utero have much higher levels of viremia than infants infected through other routes or adults, and the progression to AIDS and death are accelerated with approximately 25% of infected untreated infants dying in the first year of life. Reasons for this are not fully understood; however, one contributing dynamic is the highly active thymic function in infancy that provides large pools of CD4Tcells for viral attachment. Thymic function is very robust in infants resulting in large T cell pools in infants and young children, and thereafter it begins to decline by so that by early adulthood thymic output is significantly compromised. The large numbers of CD4 T cells, coupled with an immature immune response to high levels of viremia likely contributes to poorer prognosis in this group (Abel, 2009; Tobin & Aldrovandi, 2013). Increased thymic function in older children on therapy, however, allows for more rapid and robust immune recovery, replacing depleted T cell populations with large influxes of recent thymic emigrants which display a naïve phenotype (Tobin & Aldrovandi, 2013).

Pediatric HIV in Ethiopia

It is estimated that there are over 2.1million children worldwide under the age of 15 who are infected with HIV and of these 2.2 million are estimated to be living in sub-Saharan Africa (Siberry, 2014). It is estimated that there are approximately 140,000 children under the age of 15 living with HIV in Ethiopia with a projected 4,000 new cases presenting yearly and over 8,000 deaths from AIDS occurring each year (Fitun, 2011). Children with HIV in developing countries such as Ethiopia also encounter other challenges that impact clinical outcomes including endemic intestinal helminths,

malnutrition, and greater exposure to pathogens (Duggal, Chugh, & Duggal, 2012; ICF International, 2012; Mengist, Taye, & Tsegaye, 2015; Nyantekyi et al., 2010; Taye, Shiferaw, & Enquselassie, 2010; Yami, Mamo, & Kebede, 2011). While ART has had positive impacts in reducing viral loads and increasing CD4 counts, numerous children still demonstrate a suboptimal clinical picture with chronic diarrhea, compromised immune function, and significant stunting as well as cognitive delays (Asfawesen, Solomie, Bisirat, Mebratu, & Rahlenbeck, 2011; Taye, Shiferaw, & Enquselassie, 2010).

A national survey of child nutrition conducted in Ethiopia in 2011 reported 44% of their child population as being malnourished (ICF International, 2012). This percentage is expected to be higher in the pediatric HIV population as malnutrition is amplified in individuals with HIV due to poor appetite, diarrhea and poor absorption and utilization of nutrients (Duggal, Chugh, & Duggal, 2012; Taye et al., 2010). Investigations on nutritional status of HIV+ children in Ethiopia treatment with z-scores remaining < -1 after initiation of ART (Asfawesen, et al., 2011; Taye et al., 2010). Undernutrition alone is a known cause of immune deficiency and malnutrition has been correlated with disease progression and poor immune reconstitution in HIV, even following the initiation of ART (Duggal et al., 2012).

Microbial Translocation in Pediatric HIV

As in adults, the gastrointestinal tracts of infants and children were recognized early on in the AIDS epidemic to be severely affected by HIV as children developed chronic intractable diarrhea without the identification of specific pathogens. Malabsorption with malnutrition and wasting were common symptoms with advancing disease in the early days of HIV (Guarino, Bruzzese, De Marco & Buccigrossi, 2004).

The development of the intestinal tract is complete before birth; however, functional maturity is not complete until approximately 6 months of age, and the microbiota of children does not mirror adults until 2-3 years of age (Tobin & Aldrovandi, 2013). Infants contract the virus before the functional status of the intestinal tract is complete, and the damage that occurs in early infection may result in fibrosis and irrevocable damage to both structure and function of the GI system (Wallet et al., 2010).

In one of the first investigations into microbial translocation in children, Wallet, et.al. (2010), assayed markers of microbial translocation and immune activation along with CD4 and viral loads in 33 HIV+ children ages 2 months to 17.7 years of age who were enrolled in a clinical trial evaluating combination ART including protease inhibitors. Analyses were performed on stored samples collected pretreatment and at intervals up to 96 weeks post initiation of therapy. These were compared against 14 HIV- controls, ages 4 months -15 years, who were recruited for this project. All of the infants demonstrated LPS in plasma samples however by age 2 the healthy controls had cleared LPS; whereas, HIV+ subjects of all ages continued with elevated levels of LPS as well as sCD14 (median 931.8 ng/ml) well above controls throughout the 96 week period ($p < 0.05$ and < 0.0001 respectively)(Wallet et al., 2010). The presence of LPS in the plasma of all infants was not surprising as it is known that tight junctions in the gut epithelium of infants are more permeable to allow for antigen sampling and mucosal immune development (Abel, 2009) and LPS exposure in early infancy does not elicit immune activation (Tobin & Aldrovandi, 2013). EndoCab levels were reduced in all the HIV+ children in Wallet's trial but did not correlate to LPS levels and sCD27, a general marker of immune activation, was elevated in all HIV+ groups. Of note, tumor necrosis

factor (TNF), a mediator of systemic inflammation, was only detected in 8 of 33 of the HIV+ children pretreatment, but levels in these children remained elevated throughout the trial period. The authors concluded that microbial translocation, although seemingly independent of immune activation in this trial, is not responsive to ART and in combination with chronic immune activation will likely accelerate immune senescence and lead to the development of increased non-HIV morbidity and mortality in this population (Wallet et al., 2010). The authors also noted that the lack of decline of LPS and microbial translocation markers after the initiation of ART in these children may indicate "irreversible intestinal fibrosis" due to early viremia during the time of gut remodeling that occurs in young children (Wallet et al., 2010). Intestinal fibrosis has been noted in adults with HIV as well but the early development in children may have greater long-term implications.

Fifty-four HIV+ and 22 HIV exposed but uninfected (HEU) infants from South Africa were evaluated for microbial translocation pre and post initiation of ART. The first 6 months of life were designated as the 1st semester, and the 2nd semester designated months beyond 6 months and up to a year. Twenty HIV+ infants with delayed initiation of ART (after 12-weeks) were considered Group 1 while 35 HIV+ infants who had been started on ART in the first 6-12 weeks of life were considered Group 2. Twenty-two HIV negative infants born to HIV+ mothers served as age matched controls. All the infants started with CD4 percentages ≥ 25% and all were started on ART by 6 months. Measures included LPS, sCD14, LBP, and EndoCab as well as CD4 counts, viral loads and cell surface activation markers. LPS levels were higher during the first semester in both the untreated group and the treated group of HIVs+ infants compared with the HIV-

controls (p = 0.03 and 0.01 respectively), however elevated levels of LPS were not detected in any infants at the conclusion of the 2nd semester. All of the LPS “control” molecules, (sCD14, LBP and EndoCab) as well as T cell activation markers, however, were significantly elevated in the delayed treatment group but not the treated group and remained elevated through the 2nd semester. In this small pilot, significance was not noted between LPS and LPS control molecules or T cell activation at the first time point; however, CD8+ T cell activation was positively correlated with viral load (p= 0.002) and with LPS control molecules at the end of the 2nd semester (Papasavvas et al., 2011). Delayed initiation in this trial was considered treatment initiation by 6 months of age which in many developing countries would still be considered early initiation. The non-detectable levels of LPS by age one in both groups would support a positive impact on HIV enteropathy of early initiation of ART; however, the presence of LPS control molecules in both groups that did not decline significantly challenges this conclusion.

In a third trial, stored plasma samples from 85 clinically stable HIV children ages 2-17 who had been enrolled in the Pediatric AIDS Clinical Trial Group (PACTG) 338, were used to evaluate the presence and impact of microbial translocation and immune activation. Comparisons were made between week 0 and week 44 after the initiation of a protease inhibitor-based ART regimen using markers of microbial translocation (LPS, sCD14, S16rDNA), T cell activation markers (CD38, HLA-DR), as well as CD4 counts and viral loads. Samples were sub grouped into viral responders (VR) vs viral failures (VF) based on a cutoff of <400 copies/ml or >400 copies/ml. While T cell activation markers (CD38, HLA-DR) decreased in the VR group by week 44, both groups maintained significantly elevated levels of LPS, sCD14, and S16rDNA compared to

controls. In this trial sCD14 correlated positively with LPS levels at enrollment (p = 0.005) and was positively correlated with S16rDNA in both VR and VF over the course of the trial. The authors concluded that sCD14 is a "consistent marker" of direct immune activation related to microbial translocation and that levels of sCD14 were not impacted by ART, indicating ongoing intestinal pathology in this group of children (Pilakka-Kanthikeel et al., 2012).

Immune Activation and Comorbidities in Pediatric HIV

It's hard to project what the impact of chronic immune activation and inflammation will have on HIV+ children, however, a few recent studies are raising concern about the early development of comorbidities in children. Several trials have pointed to ART, and especially regimens containing protease inhibitors(PI), as being responsible for deleterious effects on lipid metabolism both in children and adults (Duprez et al., 2012, 2012; Guzmán-Fulgencio et al., 2011; Lainka et al., 2002), however, several of these trials have also reported other markers of inflammation and cardiovascular risk, such as CRP, IL-6 and D-dimers elevated in pediatric cohorts, and unrelated to PI use (Guzmán-Fulgencio et al., 2011; Lainka et al., 2002; Leonard & McComsey, 2005; Pilakka-Kanthikeel, Kris, Selvaraj, Swaminathan, Pahwa, 2014).

The Pediatric HIV/AIDS Cohort Study (PHACS), evaluated 226 HIV+ children and 140 HIV exposed uninfected (HEU) children ages 7-16, from the US and Puerto Rico, to determine comparative risk for cardiovascular disease (CVD) in a pediatric HIV cohort (Miller et al, 2012). All HIV+ children were well-controlled on ART with 65% having viral loads ≤400copies/ml and 75% had CD4 counts > 500mm^3. Measures included markers of inflammation (C reactive protein (CRP), IL-6 and monocyte

chemoattractant protein-1 (MCP-1), endothelial and vascular dysfunction (soluble vascular cell adhesion molecule-1 (sVCAM), soluble intracellular cell adhesion molecule -1 (sICAM), and E-selectin), and coagulation dysfunction (soluble P-selectin, fibrinogen) as well as fasting total cholesterol, high density lipid (HDL) cholesterol, low density lipid (LDL) cholesterol, triglycerides, glucose, insulin and anthropometrics. This group demonstrated that in spite of having lower body mass indexes (BMIs) and percentage of body fat, HIV+ children compared to HIV negative controls had significantly higher biomarkers of inflammation, coagulation and vascular dysfunction. They did not note a strong association of these markers with ART use, however they did find that higher viral loads were correlated with many of their markers (Miller et al., 2012).

DiMeglio, et al., (2013), performed DEXA scans (total body and lumbar spine) on 510 children, ages 7-15, enrolled in the Adolescent Master Protocol arm of the Pediatric HIV/AIDS Chohort Study (PHACS). The 350 HIV+ children had lower height, weight and BMI Z-scores than the 160 HEU controls and had higher incidence of total body Z-scores of < 2 (7 vs 1%, p=0.008) and lumbar spine Z-scores of < -2 (4 vs 1% p=0.08). The Z-score is a measure of how many standard deviations a measure is from an established population standard. The impact of immune activation on bone health in children is very concerning as childhood is the period of rapid bone growth and establishment of bone density which peaks in early adulthood and declines thereafter. The impacts of HIV on bone health are two fold with both the premature development of osteoporosis (indicating bone resorption) and the increased risk for avascular necrosis (loss of blood supply to bone) which results in weakened bone and fractures (DiMeglio

et al., 2013).

More investigations into the correlations of chronic immune activation and the development of comorbidities in pediatric HIV are being designed and conducted with most pointing in the direction of early development of many age-related diseases (Dimock et al., 2011; Eckard, Fowler, Haston, & Dixon, 2016; Leonard & McComsey, 2005; Stephensen, Marquis, Douglas & Wilson (2005); Sudjaritruk & Puthanakit, 2015). This begs for attention and the development of appropriate interventions.

Current Interventions for HIV Enteropathy

To date only a few interventions have been trialed for their ability to impact HIV enteropathy. Selamer, a phosphate binding drug which is used for renal insufficiency, also binds LPS in these patients and results in lowered sCD14 as well as cardiac risk markers. The use of Sevelamer for HIV enteropathy looked promising when trialed in SIV pigtail macaques showing “dramatic reductions” in LPS and sCD14 as well as on markers of coagulation (Kristoff et al., 2014); however, a trial conducted by Sandler, et al in 40 HIV adults with early-onset disease showed no significant decline in LPS, sCD14, LBP or endotoxin as well as no decline in markers of inflammation or T cell activation (Sandler et al., 2014). Rivaximin, a non-absorbable antibiotic, used for traveler’s diarrhea, irritable bowel disease, and cirrhosis, showed only minimal impact on measures of microbial translocation when given alone (Tenorio et al., 2015). Rivaximin demonstrated somewhat better impact in an SIV model of enteropathy when partnered with sulfasalazine, an anti-inflammatory drug used in inflammatory bowel disease, however, this combination to date has not been trialed in humans (Pandrea et al., 2016). A distinct problem with each of these pharmaceutical interventions is the

increased “pill burden” on top of combination HIV regimes which also increases the prevalence of adverse events (Hatano, 2013).

A novel intervention has been oral supplementation with serum bovine immunoglobulins. Serum-derived bovine immunoglobulin isolate (SBI) is salvaged from plasma obtained from slaughter houses which has been hygienically processed into an USDA approved edible powder, high in protein and immunoglobulins (Petschow, 2014). The field of animal husbandry has used plasma-derived protein concentrates (PPC) in young animals to attenuate intestinal inflammation and enhance weight gain (Pierce, Cromwell, Lindemann, Russell, & Weaver, 2005). A small trial was conducted with 8 HIV+ individuals who had moderate to severe symptoms of HIV enteropathy with increased numbers of watery diarrheal stools who incorporated SBI into their diet daily and reported significant improvements in GI symptom scores ($p = 0.008$), as well as significant increases in lamina propria CD4+ T- cells ($p = 0.016$) (Asmuth et al., 2013).

In a trial conducted in SIV+ macaques, Klatt et al. (2015) evaluated the efficacy of ART in combination with probiotics and prebiotics (PP), compared with that of ART alone. Animals in the PP-supplemented group had a greater than 2-fold increase in colonic CD4 cells, with higher percentages of Th17 cells (involved in defense against colonic bacteria and epithelial repair), increased functionality of colonic antigen presenting cells, increased levels of transforming growth factor beta, and significantly reduced lymphoid follicle fibrosis. Lymphoid follicle fibrosis has been shown to be associated with persistent immune abnormalities including failure to mount adequate responses to post-ART vaccines (Klatt et al., 2013). The addition of probiotics and prebiotics to the diets of HIV individuals has also been evaluated in several small trials

and has shown promise by repeatedly demonstrating significance in their ability to decrease measures of microbial translocation and enhance the microbiota which may in turn improve the epithelial barrier (Gori et al., 2011; Serrano-Villar et al., 2016; Stiksrud et al., 2015).

All of these interventions are worth further exploration, as are other innovative therapies that are affordable, sustainable and hold low-risk potential for use in pediatric populations in resource-poor countries where most of the pediatric HIV is seen.

Nutrition and Gut Mucosal Health

Although there are still many unanswered questions, it has become increasingly apparent that diet has significant impacts on the gut microbiota and that commensal bacteria engage in significant crosstalk with gut epithelium which in turn has impacts on mucosal integrity and immunity (Artis, 2008; X. Xu, Xu, Ma, Tang, & Zhang, 2013; Z. Xu & Knight, 2015). As a result, various plant and nutritional substances are beginning to be evaluated in models of inflammatory bowel disease and have shown promise in reducing inflammation and aiding tissue healing. The benefits of these substances are largely attributed to enhancements of the microbiota which, in turn, has immunomodulatory impacts on the gut milieu (Rajilić-Stojanović, 2013).

Whey protein and aloe are both nutritional supplements that have been shown to support the gut microbiota (Faure et al., 2006; Yagi & Yu (2015) and tissue healing Abdel-Selam, Ebaid, Al-Tamimi, & Alhazza, 2016; Davis et al., 1994), and have also been documented to have anti-inflammatory (Davis, Donato, Hartman & Haas, 1994; Krissansen, 2007) and antioxidant properties (Hu, Xu, & Hu, 2003; Micke, Beeh, & Buhl, 2002). In addition, both have been trialed in models of colitis and reported to influence

positive outcomes (Park, Kwon, & Sung, 2011; Sprong, Schonewille & van der Meer, 2010). Therefore, these 2 nutritional support products are good candidates for evaluation in HIV enteropathy.

Whey Protein

Whey protein is a byproduct of cheese production. It is processed by dehydration techniques to maintain high levels of non-denatured proteins as well as hydrolyzed free essential and non-essential amino acids (Smithers, 2008). It has been shown to be a well-tolerated, easily digestible and affordable source of protein for nutritional supplementation and has also shown potential for reducing inflammation and restoring glutathione levels in models of inflammatory bowel disease and HIV.

In their study using a murine model, Sprong et al. (2010) found that dietary supplementation with whey protein protected rats against dextran sulfate sodium–induced colitis by reducing levels of IL-1β, calprotectin, and inducible nitric oxide, while increasing levels of Lactobacilli and Bifidobacterium in the gut. In a case study report, Bounous, Baruchel, Falutz & Gold (1993), documented weight gains and elevation of glutathione levels in 3 HIV+ men who supplemented their diets with whey protein for 12 weeks. A 6-month trial of whey protein supplementation in HIV+ participants also found significant increases ($p = 0.033$) in plasma glutathione levels from baseline; however, no significant increases in weight or CD4 counts were noted (Micke et al., 2002). In this trial 2 different brands of whey protein were being evaluated with one resulting in significant changes in glutathione and one not reaching significance and attributed to differences in product quality.

Children with HIV have higher nutrient needs and the WHO recommends adding

protein supplementation to the diet of malnourished HIV+ children (Willumsen, 2011). A whey protein drink was used in this trial to provide supplemental protein and amino acids to support mucosal healing and to boost antioxidant reserves. A chocolate drink was anticipated to be well accepted and to provide a suitable carrier in which to mix powdered aloe.

Aloe Vera

Aloe Vera is a succulent member of the lily family that has been used by multiple cultures for thousands of years and for numerous medicinal purposes (Aguayo & McAnallley, 2009; Reynolds & Dweck, 1999; Sierra-Garcia, Castro-Rios, Gonzalez-Horta, Lara-Arias, & Chavez-Montes, 2014). The aloe leaf is composed of an outer green rind covering the inner clear pulp. In between the outer rind and inner gel is a sticky yellow sap known as aloin which is rich in anthraquinones. This latex sap can produce a laxative effect and is responsible for “toxic” properties sometimes attributed to aloe so is generally removed from commercial products (Aguayo, 2009). The gel is largely composed of high molecular weight polymers of β (1,4) linked mannose, β (1,4) linked glucose and α (1,6) linked galactose in an approximate ratio of 31:1:1 which are randomly acetylated at C2 or C3 (Femenia, García-Pascual, Simal, & Rosselló, 2003) .The linear high mannose polymer has been named acemannan and is considered to be aloe’s main health-promoting component (Femenia et al., 2003; Aguayo & McAnalley, 2009). Aloe preparations that are processed to preserve the acemannan content are good sources of exogenous mannose. Other components in small amounts are found in whole leaf preparations including vitamins, minerals, amino acids, and antioxidants and other sugars (Reynolds & Dweck, 1999).

The importance of mannose in cellular physiology is highlighted by the presence of mannose transporters on several cell types throughout the body including intestinal epithelial cells (enterocytes) and the ability of these transporters to facilitate mannose uptake even in the presence of much higher concentrations of other sugars including glucose. (Alton et al., 1998; Duran, 2004). Alton, et al. (1998), assessed blood levels of mannose from 35 different species of animals and found that mannose was present in all with a mean mannose concentration being 75 µM. For humans the average was determined to be 55µM. While little is known at this time about sources of mannose, their studies with hepatoma cell glycoproteins concluded that 75% of the mannose incorporated into glycoprotein structures is from exogenous mannose, likely from both salvage pathways and dietary sources. The remaining 25% is likely derived from the conversion of glucose which was previously thought to provide all mannose needs. (Alton et al., 1998)

Aloe is one of at least 35 different plant species whose polysaccharides are being explored as polysaccharide biological response modifiers (PBRM) due to their ability to stimulate and modify function of immune cells, in particular macrophage and dendritic cells (Leung et al., 2004; Schepetkin & Quinn, 2006; Song et al., 2002). This includes enhancing phagocytic activity, increasing production of reactive oxygen species (ROS) and nitric oxide (NO), as well as upregulating expression of activation markers and secretion of cytokines and chemokines (Leung, Liu, Koon, & Fung, 2006; Liu et al., 2006). In addition, they have been shown to stimulate proliferation and differentiation within these cell lines (Egger et al., 1996; Lee et al., 2001; Leung et al., 2006). The ability of plant polysaccharides to elicit an immune response is attributed to

binding of cell receptors and plasma proteins, including pattern recognition receptors (PRRs) and carbohydrate binding proteins (CBPs), which are able to recognize and bind saccharides on PBRMs (Leung et al., 2006; Schepetkin & Quinn, 2006).

The high mannose content of aloe polysaccharides allows them to be recognized by mannose receptors (MR). Mannose receptors are PRR which contain a cysteine rich (CR) domain that binds sulfated carbohydrates, a fibronectin II domain which binds collagen and multiple C-type lectin-like domains (CTLDs) which recognize mannose, fucose, and *N*-acetylglucosamine (East, 2002; Martinez-Pomares, 2012). They had originally been identified on macrophage and dendritic cells but more recently have been identified on several other cell types including non-vascular endothelium, tracheal smooth muscle, mesangial and epithelial cells from various locations including the cervix and small intestine (Fanibunda, Modi, Gokral, & Bandivdekar, 2011; Martinez-Pomares, 2012; Schepetkin & Quinn, 2006; Su et al., 2009). Epithelial cells, once thought to only provide a physical barrier have now been shown to express various PRRs and have demonstrated the ability to launch both innate and adaptive immune responses as well as suppress inflammation and maintain immune homeostasis in the gut (Artis, 2008; Philpott, Girardin, & Sansonetti, 2001).

The hypothesized mechanisms of how aloe polysaccharides may impact HIV enteropathy and ultimately HIV outcomes are outlined below:

1) In the intestinal tract, aloe polysaccharide chains are cleaved by mannosidases and other enzymes into smaller units. The “digestion” of aloe polysaccharides provides a time released fuel source for enterocytes as well as release of monosaccharides for absorption and utilization in glycoforms including glycoproteins in the glycocalyx

(Aguayo & McAnalley, 2009). This may enhance the physical barrier of the glycocalyx as well as support enterocyte health.

2) Multimers of aloe polysaccharide chains in the gut lumen may interact directly with mannose receptors or other PRR on the gut epithelium triggering an immune response and tissue repair. Wound studies have demonstrated increased proliferation of both fibroblasts and epithelial cells in response to exposure to aloe polysaccharides (Chen et al., 2005, Yao et al, 2006). In this way damaged enterocytes could be renewed by proliferating cells.

3) Similarly, its plausible that these molecules may be trancytosed through enterocytes or intestinal M cells or bind with and stimulate dendritic cells sampling the gut lumen (Varol, Zigmond, & Jung, 2010). Research with a radiolabeled polysaccharide from mushrooms showed that large molecular weight fractions (>10,000 Da) were able to be transported intact across the intestinal lining following oral dosing although the specific mechanism was not determined. (Tsukagoshi et al., 1984) It is therefore plausible that mannose polymers may gain direct access to the lamina propria and bind to resident fibroblast, macrophage and dendritic populations. Fibroblasts have been shown to express increased quantities of growth factors that assist epithelial repair in response to binding of mannose-6-phosphate to receptors on their surface (Davis et al., 1994). Macrophage in the lamina propria may be stimulated to increase phagocytosis and expression of tissue repair chemokines; however, this tolerogenic population does not express inflammatory cytokines (Sheikh & Plevy, 2010). Dendritic cells may be stimulated and provide increased surveillance and clearance of microbial products that cross the mucosal barrier or initiate an adaptive response leading to healing of mucosal

surfaces.

4)The undigested portions of polysaccharide chains are fermented by commensal bacteria, resulting in short-chain fatty acids (SCFAs), including butyrate, which are also major fuel sources for the gut epithelium and are able to trigger anti-inflammatory signaling (den Besten et al., 2013).

In clinical trials SCFAs have been shown to be of benefit in the treatment of Crohns disease and other inflammatory bowel diseases through attenuation of inflammatory cytokines (Di Sabatino et al., 2005; Xu,X. et al., 2013). Aloe polysaccharides have been shown to attenuate inflammatory cytokines in models of colitis (Koetzner, Grover, Boulet & Jacoby, 2010). Budai, Varga, Milesz, Tozser and Benko (2013) induced primary and THP1 macrophages in vitro with lipopolysaccharide in the presence or absence of different concentrations of aloe and showed significant reductions in IL1-β, IL-6, IL-8, and TNFα in a dose–dependent manner. An investigation with a murine model of colitis found that rats fed diets incorporating various aloe preparations for 2 weeks followed by a 1-week induction of colitis with dextran sulfate sodium had significantly lower expression of colonic mucosal TNF-α and IL-1β than controls ($p < 0.05$) (Park et al., 2011). Langemead et al. (2004), conducted a 4-week blinded randomized trial with 44 individuals ages 18-80 with mild to moderately active ulcerative colitis based on a Simple Clinical Colitis Activity Index (SCCAI). Thirty participants were randomized to ingest a flavored aloe gel drink twice daily while 14 were randomized to a placebo group receiving a similar flavored drink. All previous medication regimens were continued throughout the trial. Participants completed the index at enrollment and underwent rigid sigmoidoscopy where mucosal surfaces were

scored using a Baron scale. Rectal biopsies were also taken for histologic scoring. Participants also completed the Inflammatory Bowel Disease Questionnaire (IBDQ), physicians completed a global assessment interview and blood work was collected consisting of hemoglobin, platelet count, serum albumin, CRP, and erythrocyte sedimentation rate (ESR). Study variables were repeated at the conclusion of 4 weeks. SCCAI scores were used to group participants such that a score of ≤ 2 indicated remission and a score decrease of ≥ 3 indicated improvement. Baron scores on sigmoidoscopy and Saverymuttu scores from histology of ≤1 indicated remission. Groups were similar based on outcome variables at enrollment. At study conclusion physician global assessments were unchanged in both groups. The scores from the IBDQ were unchanged in the aloe group but improved in the placebo group. The median SCCAI scores in the Aloe Vera group were significantly decreased (p=0.01) but not in the placebo group. Sigmoidoscopy scores were not significantly changed in either group, however, histology scores were significantly improved in the aloe group (p=0.03). No significant change was seen in blood work in either group. Forty seven percent of the aloe group showed clinical response versus fourteen percent in the placebo group with an odds ratio (OR) of 5.3 which study authors report being similar to a meta-analysis with Mesalazine (Langmead et al., 2004).

Aloe polysaccharides have also demonstrated antioxidant potential. A trial to evaluate this found that aloe's radical-scavenging ability was higher than that of α-tocopherol and butylated hydroxytoluene, two well-recognized antioxidants (Hu et al., 2003). In this trial aloe plants of different ages (2, 3 and 4-year-old plants) were used and while all showed good antioxidant activity there were differences noted based on

the age of plants with the 3-year-old plants displaying the highest antioxidant activity.

A small clinical trial in Nigeria enrolled 10 HIV+ treatment naive women who were given a fresh aloe “gruel” daily for one year. Outcomes of CD4 counts and weight were compared with 20 age-matched controls who were started on standard ART. Both groups had significant gains in CD4 counts and weight and although the ART group had higher CD4 counts than the supplemented group at the end of the year, the difference was not statistically significant (Olatunya, Olatunya, Anyabolu, Adejuyigbe, & Oyelami, 2012).

Innovation

The reported properties of whey protein and aloe make them good candidates to consider for addressing HIV enteropathy. Each of these nutritional support products has been shown to be beneficial to individuals with HIV (Bounous, Baruchel, Falutz, & Gold, 1993; Olatunya et al., 2012), however, they have not been evaluated in combination and specifically on measures of HIV enteropathy.

The interventions trialed in this research are not put forth as potential treatments for HIV but rather as nutritional support strategies to support a healthy gut milieu which, in turn, supports physiologic healing and immune regulation processes. Addressing the ongoing pathology in the gut was hypothesized to have significant impacts on HIV disease progression and the development of comorbidities. Healing of gut mucosal tissues may also result in better absorption and assimilation of nutrients.

The fact that both of these nutritional support products have the potential to be manufactured locally in Ethiopia was another reason for exploring their use. Several species of aloe are indigenous to East Africa and Ethiopia specifically and large scale

dairy farming incorporating cheese production is growing in Ethiopia.

Mass treatment for intestinal parasites in HIV populations living in endemic areas has been recommended to address contributors to immune activation and disease progression by helminth researchers (Borkow & Bentwich, 2006), but this has not been evaluated in HIV+ children in Ethiopia. Antihelminth treatment was incorporated as a 3rd intervention in this trial. This intervention alone was not intended to address HIV enteropathy but rather to remove one source of immune activation, which could confound data and contribute to the development of comorbidities.

The impact of these 3 interventions on measures of HIV enteropathy was assessed using a clinical trial with HIV+ children living in Addis Ababa, Ethiopia. This trial evaluated the impact of mass helminth treatment combined with daily dietary supplementation with an aloe-enriched whey protein drink, compared with mass helminth treatment and daily intake of an isocaloric drink. Measures of HIV enteropathy, specifically enterocyte damage, microbial translocation and immune activation as well as measures of immune reconstitution, and clinical status, were assessed pre and post supplementation.

REFERENCES

REFERENCES

Abdel-Selam, B., Ebaid, H., Al-Tamimi, J., & Alhazza, I. (2016). Enhancement of Wound Healing by Un-denatured Camel Whey Proteins in Protein Malnourishded Mice. *Pakistan J Zool*, *48*(1), 1–9.

Abel, K. (2009). The rhesus macaque pediatric SIV infection model - a valuable tool in understanding infant HIV-1 pathogenesis and for designing pediatric HIV-1 prevention strategies. *Current HIV Research*, *7*(1), 2–11.

Agauyo, E., McAnalley,W.(Eds)(2009), *The Science Behind Aloe: The Healing Plant* (1st Ed), Dallas, TX., William McAnalley. ISBN 10: 0615263941

Alton, G., Hasilik, M., Niehues, R., Panneerselvam, K., Etchison, J. R., Fana, F., & Freeze, H. H. (1998). Direct utilization of mannose for mammalian glycoprotein biosynthesis. *Glycobiology*, *8*(3), 285-295. https://www.ncbi.nlm.nih.gov/pubmed/9451038

Appay, V., & Sauce, D. (2008). Immune activation and inflammation in HIV-1 infection: causes and consequences. *The Journal of Pathology*, *214*(2), 231–241. https://doi.org/10.1002/path.2276

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature Reviews. Immunology*, *8*(6), 411–420. https://doi.org/10.1038/nri2316

Asfawesen, G., Solomie, D., Bisirat, T., Mebratu, B., & Rahlenbeck, S. (2011). Outcome in a pediatric cohort receiving ART in Addis Abeba, Ethiopia. *Acta Paediatrica*, *100*(8), 1164–7.

Asmuth, D. M., Ma, Z.-M., Albanese, A., Sandler, N. G., Devaraj, S., Knight, T. H., … Miller, C. J. (2013). Oral serum-derived bovine immunoglobulin improves duodenal immune reconstitution and absorption function in patients with HIV enteropathy: *AIDS*, *27*(14), 2207–2217. https://doi.org/10.1097/QAD.0b013e328362e54c

Borkow, G. & Bentwich, Z. (2006, November), HIV and helminth co-infection: is deworming necessary? *Parasite Immunology,* 28(11), 605-612. http://doi.wiley.com/10.1111/j.1365-3024.2006.00918.x

Blanco, J. (2012, December). Chronic immune activation, causes and pathogenic mechanisms in HIV infection - Event - ISGLOBAL. Retrieved September 7, 2013, from http://www.isglobal.org/-/chronic-immune-activation-causes-and-pathogenic-mechanisms-in-hiv-infection

Blodget, E., Shen, C., Aldrovandi, G., Rollie, A., Gupta, S. K., Stein, J. H., & Dubé, M. P. (2012). Relationship between Microbial Translocation and Endothelial Function in HIV Infected Patients. *PLoS ONE*, *7*(8), e42624. https://doi.org/10.1371/journal.pone.0042624

Bounous, G., Baruchel, S., Falutz, J., & Gold, P. (1993). Whey proteins as a food supplement in HIV-seropositive individuals. *Clinical and Investigative Medicine. Médecine Clinique Et Experimentale*, *16*(3), 204–209.

Brenchley, J. M., & Douek, D. C. (2008). HIV infection and the gastrointestinal immune system. *Mucosal Immunology*, *1*(1), 23–30. https://doi.org/10.1038/mi.2007.1

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., … Douek, D. C. (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature Medicine*, *12*(12), 1365–1371. https://doi.org/10.1038/nm1511

Brown, T. T., & Qaqish, R. B. (2006). Antiretroviral therapy and the prevalence of osteopenia and osteoporosis: a meta-analytic review. *AIDS (London, England)*, *20*(17), 2165–2174. https://doi.org/10.1097/QAD.0b013e32801022eb

Budai, M. M., Varga, A., Milesz, S., Tőzsér, J., & Benkő, S. (2013). Aloe vera downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages. *Molecular Immunology*, *56*(4), 471–479. https://doi.org/10.1016/j.molimm.2013.05.005

Bunders, M. J., van der Loos, C. M., Klarenbeek, P. L., van Hamme, J. L., Boer, K., Wilde, J. C. H., … Kuijpers, T. W. (2012). Memory CD4+CCR5+ T cells are abundantly present in the gut of newborn infants to facilitate mother-to-child transmission of HIV-1. *Blood*, *120*(22), 4383–4390. https://doi.org/10.1182/blood-2012-06-437566

Burdo, T. H., Lo, J., Abbara, S., Wei, J., DeLelys, M. E., Preffer, F., … Grinspoon, S. (2011). Soluble CD163, a Novel Marker of Activated Macrophages, Is Elevated and Associated With Noncalcified Coronary Plaque in HIV-Infected Patients. *Journal of Infectious Diseases*, *204*(8), 1227–1236. https://doi.org/10.1093/infdis/jir520

Canipe, A., Chidumayo, T., Blevins, M., Bestawros, M., Bala, J., Kelly, P., … Koethe, J. R. (2014). A 12-week longitudinal study of microbial translocation and systemic inflammation in undernourished HIV-infected Zambians initiating antiretroviral therapy. *BMC Infectious Diseases*, *14*, 521. https://doi.org/10.1186/1471-2334-14-521

Castello-Branco, L. R. R., Lewis, D. J. M., Ortigão-de-Sampaio, M. B., & Griffin, G. E. (1996). Gastrointestinal immune responses in HIV infected subjects. *Memórias*

Do Instituto Oswaldo Cruz, *91*(3), 363–366.

Chen, X., Wu, B., Jiang, Q., Wang, S., Huang, L., & Wang, Z. (2005). [Influence of polysaccharide from Aloe vera on the proliferation of the human epithelial cells cultured in vitro]. *Zhonghua Shao Shang Za Zhi = Zhonghua Shaoshang Zazhi = Chinese Journal of Burns*, *21*(6), 430–433.

Coaccioli, S., Crapa, G., Fantera, M., Del Giorno, R., Lavagna, A., Standoli, M. L., … Puxeddu, A. (2010). Oxidant/antioxidant status in patients with chronic HIV infection. *La Clinica Terapeutica*, *161*(1), 55–58.

Conway, B., & Tossonian, H. (2014). HIV/AIDS-A Model of Chronic Oxidative Stress and Immune Activation. In *Systems Biology of Free Radicals and Antioxidants* (pp. 3217–3238).

Dandekar, S. (2007). Pathogenesis of HIV in the gastrointestinal tract. *Current HIV/AIDS Reports*, *4*(1), 10–15.

Davis, R. H., Donato, J. J., Hartman, G. M., & Haas, R. C. (1994). Anti-inflammatory and wound healing activity of a growth substance in Aloe vera. *Journal of the American Podiatric Medical Association*, *84*(2), 77–81. https://doi.org/10.7547/87507315-84-2-77

Deeks, S. G. (2011). HIV Infection, Inflammation, Immunosenescence, and Aging. *Annual Review of Medicine*, *62*(1), 141–155. https://doi.org/10.1146/annurev-med-042909-093756

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D.-J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *The Journal of Lipid Research*, *54*(9), 2325–2340. https://doi.org/10.1194/jlr.R036012

Di Sabatino, A., Morera, R., Ciccocioppo, R., Cazzola, P., Gotti, S., Tinozzi, F. P., … Corazza, G. R. (2005). Oral butyrate for mildly to moderately active Crohn's disease. *Alimentary Pharmacology & Therapeutics*, *22*(9), 789–794. https://doi.org/10.1111/j.1365-2036.2005.02639.x

DiMeglio, L. A., Wang, J., Siberry, G. K., Miller, T. L., Geffner, M. E., Hazra, R., … Jacobson, D. L. (2013). Bone mineral density in children and adolescents with perinatal HIV infection: *AIDS*, *27*(2), 211–220. https://doi.org/10.1097/QAD.0b013e32835a9b80

Dimock, D., Thomas, V., Cushing, A., Purdy, J. B., Worrell, C., Kopp, J. B., … Hadigan, C. (2011). Longitudinal assessment of metabolic abnormalities in adolescents and young adults with HIV-infection acquired perinatally or in early childhood. *Metabolism*, *60*(6), 874–880. https://doi.org/10.1016/j.metabol.2010.08.007

Duggal, S., Chugh, T. D., & Duggal, A. K. (2012). HIV and Malnutrition: Effects on Immune System. *Clinical and Developmental Immunology*, *2012*, 1–8. https://doi.org/10.1155/2012/784740

Duprez, D. A., Neuhaus, J., Kuller, L. H., Tracy, R., Belloso, W., De Wit, S., … James D. Neaton1 for the INSIGHT SMART Study Group. (2012). Inflammation, Coagulation and Cardiovascular Disease in HIV-Infected Individuals. *PLoS ONE*, *7*(9), e44454. https://doi.org/10.1371/journal.pone.0044454

Duran, J. M. (2004). D-mannose transport and metabolism in isolated enterocytes. *Glycobiology*, *14*(6), 495–500. https://doi.org/10.1093/glycob/cwh059

East, L. (2002). Characterization of Sugar Binding by the Mannose Receptor Family Member, Endo180. *Journal of Biological Chemistry*, *277*(52), 50469–50475. https://doi.org/10.1074/jbc.M208985200

Eckard, A. R., Fowler, S. L., Haston, J. C., & Dixon, T. C. (2016). Complications of Treatment in Youth with HIV. *Current HIV/AIDS Reports*, *13*(4), 226–233. https://doi.org/10.1007/s11904-016-0320-1

Egger, S. F., Brown, G. S., Kelsey, L. S., Yates, K. M., Rosenberg, L. J., & Talmadge, J. E. (1996). Hematopoietic augmentation by a beta-(1,4)-linked mannan. *Cancer Immunology, Immunotherapy: CII*, *43*(4), 195–205.

Fanibunda, S. E., Modi, D. N., Gokral, J. S., & Bandivdekar, A. H. (2011). HIV gp120 Binds to Mannose Receptor on Vaginal Epithelial Cells and Induces Production of Matrix Metalloproteinases. *PLoS ONE*, *6*(11), e28014. https://doi.org/10.1371/journal.pone.0028014

Fauci, A. (2007). Pathogenesis of HIV Disease: Opportunities for New Prevention Interventions. *Clinical Infectious Diseases,* 45(4), 206-212. http://academic.oup.com/cid/article/45/Supplement _4/S206/454681/Pathogenesis-of-HIV-Disease-Opportunities-for-New

Faure, M., Mettraux, C., Moennoz, D., Godin, J.-P., Vuichoud, J., Rochat, F., … Corthésy-Theulaz, I. (2006). Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *The Journal of Nutrition*, *136*(6), 1558–1564.

Femenia, A., García-Pascual, P., Simal, S., & Rosselló, C. (2003). Effects of heat treatment and dehydration on bioactive polysaccharide acemannan and cell wall polymers from Aloe barbadensis Miller. *Carbohydrate Polymers*, *51*(4), 397–405.

Fitun. (2011). HIV/AIDS: Estimates and projection in Ethiopia, 2011-2016. Ethiopian Central Statistical Agency. Retrieved from www.csa.gov.et/docs/EDHS 2011

Franceschi, C., & Campisi, J. (2014). Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *69*(Suppl 1), S4–S9. https://doi.org/10.1093/gerona/glu057

French, M. A., King, M. S., Tschampa, J. M., da Silva, B. A., & Landay, A. L. (2009). Serum Immune Activation Markers Are Persistently Increased in Patients with HIV Infection after 6 Years of Antiretroviral Therapy despite Suppression of Viral Replication and Reconstitution of CD4 $^{+}$ T Cells. *The Journal of Infectious Diseases*, *200*(8), 1212–1215. https://doi.org/10.1086/605890

Germolec, D. R., Frawley, R. P., & Evans, E. (2010). Markers of inflammation. *Methods in Molecular Biology (Clifton, N.J.)*, *598*, 53–73. https://doi.org/10.1007/978-1-60761-401-2_5

Gil, L., Martínez, G., González, I., Tarinas, A., Alvarez, A., Giuliani, A., … León, O. S. (2003). Contribution to characterization of oxidative stress in HIV/AIDS patients. *Pharmacological Research: The Official Journal of the Italian Pharmacological Society*, *47*(3), 217–224.

Giorgi, J. V., Hultin, L. E., McKeating, J. A., Johnson, T. D., Owens, B., Jacobson, L. P., … Detels, R. (1999). Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *The Journal of Infectious Diseases*, *179*(4), 859–870. https://doi.org/10.1086/314660

Gori, A., Rizzardini, G., van't Land, B., Amor, K. B., van Schaik, J., Torti, C., … Clerici, M. (2011). Specific prebiotics modulate gut microbiota and immune activation in HAART-naive HIV-infected adults: results of the "COPA" pilot randomized trial. *Mucosal Immunology*, *4*(5), 554–563. https://doi.org/10.1038/mi.2011.15

Guarino, A., Bruzzese, E., De Marco, G., & Buccigrossi, V. (2004). Management of gastrointestinal disorders in children with HIV infection. *Paediatric Drugs*, *6*(6), 347–362.

Guzmán-Fulgencio, M., Micheloud, D., Lorente, R., Bellón, J. M., de José Gomez, M. I., Gurbindo, M. D., … Resino, S. (2011). Cardiovascular risk markers are increased in HIV-infected children with lipodystrophy syndrome. *Journal of Infection*, *62*(3), 240–243. https://doi.org/10.1016/j.jinf.2011.01.008

Hatano, H. (2013). Immune activation and HIV persistence: considerations for novel therapeutic interventions. *Current Opinion in HIV and AIDS*, *8*(3), 211–216. https://doi.org/10.1097/COH.0b013e32835f9788

Hearps, A. C., Martin, G. E., Rajasuriar, R., & Crowe, S. M. (2014). Inflammatory Co-

morbidities in HIV+ Individuals: Learning Lessons from Healthy Ageing. *Current HIV/AIDS Reports*, *11*(1), 20–34. https://doi.org/10.1007/s11904-013-0190-8

Hu, Y., Xu, J., & Hu, Q. (2003). Evaluation of Antioxidant Potential of Aloe vera (Aloe barbadensis Miller) Extracts. *Journal of Agricultural and Food Chemistry*, *51*(26), 7788–7791. https://doi.org/10.1021/jf034255i

Hunt, P. W., Sinclair, E., Rodriguez, B., Shive, C., Clagett, B., Funderburg, N., … Lederman, M. M. (2014). Gut Epithelial Barrier Dysfunction and Innate Immune Activation Predict Mortality in Treated HIV Infection. *Journal of Infectious Diseases*, *210*(8), 1228–1238. https://doi.org/10.1093/infdis/jiu238

ICF International. (2012). Children's Health and Nutritional Status: Data from the 2011 Ethiopian Demographic and Health Survey. Calverton, Maryland, USA: ICF International. Retrieved from http://dhsprogram.com/pubs/pdf/DM25/DM25.pdf

Im, S.-A., Lee, Y.-R., Lee, Y.-H., Lee, M.-K., Park, Y. I., Lee, S., … Lee, C.-K. (2010). In vivo evidence of the immunomodulatory activity of orally administered Aloe vera gel. *Archives of Pharmacal Research*, *33*(3), 451–456. https://doi.org/10.1007/s12272-010-0315-1

Kewenig, S., Schneider, T., Hohloch, K., Lampe-Dreyer, K., Ullrich, R., Stolte, N., … Zeitz, M. (1999). Rapid mucosal CD4(+) T-cell depletion and enteropathy in simian immunodeficiency virus-infected rhesus macaques. *Gastroenterology*, *116*(5), 1115–1123.

Khaitan, A., Kravietz, A., Mwamzuka, M., Marshed, F., Ilmet, T., Said, S., … Unutmaz, D. (2016). FOXP3+Helios+ Regulatory T Cells, Immune Activation, and Advancing Disease in HIV-Infected Children. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *72*(5), 474–484. https://doi.org/10.1097/QAI.0000000000001000

Klatt, N. R., Canary, L. A., Sun, X., Vinton, C. L., Funderburg, N. T., Morcock, D. R., … Brenchley, J. M. (2013). Probiotic/prebiotic supplementation of antiretrovirals improves gastrointestinal immunity in SIV-infected macaques. *The Journal of Clinical Investigation*, *123*(2), 903–907. https://doi.org/10.1172/JCI66227

Klatt, N. R., Funderburg, N. T., & Brenchley, J. M. (2013). Microbial translocation, immune activation, and HIV disease. *Trends in Microbiology*, *21*(1), 6–13. https://doi.org/10.1016/j.tim.2012.09.001

Koetzner, L., Grover, G., Boulet, J., & Jacoby, H. I. (2010). Plant-Derived Polysaccharide Supplements Inhibit Dextran Sulfate Sodium-Induced Colitis in the Rat. *Digestive Diseases and Sciences*, *55*(5), 1278–1285. https://doi.org/10.1007/s10620-009-0848-7

Krissansen, G. W. (2007). Emerging health properties of whey proteins and their clinical implications. *Journal of the American College of Nutrition*, *26*(6), 713S–23S.

Kristoff, J., Haret-Richter, G., Ma, D., Ribeiro, R. M., Xu, C., Cornell, E., … Pandrea, I. (2014). Early microbial translocation blockade reduces SIV-mediated inflammation and viral replication. *Journal of Clinical Investigation*, *124*(6), 2802–2806. https://doi.org/10.1172/JCI75090

Kuller, L. H., Tracy, R., Belloso, W., Wit, S. D., Drummond, F., Lane, H. C., … for the INSIGHT SMART Study Group. (2008). Inflammatory and Coagulation Biomarkers and Mortality in Patients with HIV Infection. *PLoS Medicine*, *5*(10), e203. https://doi.org/10.1371/journal.pmed.0050203

Lainka, E., Oezbek, S., Falck, M., Ndagijimana, J., & Niehues, T. (2002). Marked dyslipidemia in human immunodeficiency virus-infected children on protease inhibitor-containing antiretroviral therapy. *Pediatrics*, *110*(5), e56.

Langmead, L., Feakins, R. M., Goldthorpe, S., Holt, H., Tsironi, E., De Silva, A., … Rampton, D. S. (2004). Randomized, double-blind, placebo-controlled trial of oral aloe vera gel for active ulcerative colitis. *Alimentary Pharmacology and Therapeutics*, *19*(7), 739–747. https://doi.org/10.1111/j.1365-2036.2004.01902.x

Lee, J. K., Lee, M. K., Yun, Y.-P., Kim, Y., Kim, J. S., Kim, Y. S., … Lee, C.-K. (2001). Acemannan purified from Aloe vera induces phenotypic and functional maturation of immature dendritic cells. *International Immunopharmacology*, *1*(7), 1275–1284. https://doi.org/10.1016/S1567-5769(01)00052-2

Leonard, E. G., & McComsey, G. A. (2005). Antiretroviral Therapy in HIV-Infected Children: The Metabolic Cost of Improved Survival. *Infectious Disease Clinics of North America*, *19*(3), 713–729. https://doi.org/10.1016/j.idc.2005.05.006

Leung, M. Y. K., Liu, C., Koon, J. C. M., & Fung, K. P. (2006). Polysaccharide biological response modifiers. *Immunology Letters*, *105*(2), 101–114. https://doi.org/10.1016/j.imlet.2006.01.009

Leung, M. Y. K., Liu, C., Zhu, L. F., Hui, Y. Z., Yu, B., & Fung, K. P. (2004). Chemical and biological characterization of a polysaccharide biological response modifier from Aloe vera L. var. chinensis (Haw.) Berg. *Glycobiology*, *14*(6), 501–510. https://doi.org/10.1093/glycob/cwh050

Liu, C., Leung, M. Y. K., Koon, J. C. M., Zhu, L. F., Hui, Y. Z., Yu, B., & Fung, K. P. (2006). Macrophage activation by polysaccharide biological response modifier isolated from Aloe vera L. var. chinensis (Haw.) Berg. *International Immunopharmacology*, *6*(11), 1634–1641. https://doi.org/10.1016/j.intimp.2006.04.013

Longenecker, C., Funderburg, N., Jiang, Y., Debanne, S., Storer, N., Labbato, D., … McComsey, G. (2013). Markers of inflammation and CD8 T-cell activation, but not monocyte activation, are associated with subclinical carotid artery disease in HIV-infected individuals: Immune activation and carotid disease in HIV infection. *HIV Medicine*, *14*(6), 385–390. https://doi.org/10.1111/hiv.12013

Marchetti, G., Tincati, C., & Silvestri, G. (2013). Microbial Translocation in the Pathogenesis of HIV Infection and AIDS. *Clinical Microbiology Reviews*, *26*(1), 2–18. https://doi.org/10.1128/CMR.00050-12

Martinez-Pomares, L. (2012). The mannose receptor. *Journal of Leukocyte Biology*, *92*(6), 1177–1186. https://doi.org/10.1189/jlb.0512231

Mengist, H. M., Taye, B., & Tsegaye, A. (2015). Intestinal Parasitosis in Relation to CD4+T Cells Levels and Anemia among HAART Initiated and HAART Naive Pediatric HIV Patients in a Model ART Center in Addis Ababa, Ethiopia. *PLOS ONE*, *10*(2), e0117715. https://doi.org/10.1371/journal.pone.0117715

Micke, P., Beeh, K. M., & Buhl, R. (2002). Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. *Eur J Nutr*, *41*, 12–18.

Miller, T., Borkowsky, W., DiMeglio, L., Dooley, L., Geffner, M., Hazra, R., … Pediatric HIV/AIDS Cohort Study (PHACS). (2012). Metabolic abnormalities and viral replication are associated with biomarkers of vascular dysfunction in HIV-infected children: Vascular dysfunction in paediatric HIV infection. *HIV Medicine*, *13*(5), 264–275. https://doi.org/10.1111/j.1468-1293.2011.00970.x

Neuhaus, J., Jacobs, Jr, D. R., Baker, J. V., Calmy, A., Duprez, D., La Rosa, A., … Neaton, J. D. (2010). Markers of Inflammation, Coagulation, and Renal Function Are Elevated in Adults with HIV Infection. *The Journal of Infectious Diseases*, *201*(12), 1788–1795. https://doi.org/10.1086/652749

Nyantekyi, L. A., Legesse, M., Belay, M., Tadesse, K., Manaye, K., Macias, C., & Erko, B. (2010). Intestinal parasitic infections among under-five children and maternal awareness about the infections in Shesha Kekele, Wondo Genet, Southern Ethiopia. *Ethiopian Journal of Health Development*, *24*(3). Retrieved from http://www.ajol.info/index.php/ejhd/article/view/68383

Olatunya, O. S., Olatunya, A. M., Anyabolu, H. C., Adejuyigbe, E. A., & Oyelami, O. A. (2012). Preliminary Trial of Aloe Vera Gruel on HIV Infection. *The Journal of Alternative and Complementary Medicine*, *18*(9), 850–853. https://doi.org/10.1089/acm.2010.0735

Paiardini, M., & Müller-Trutwin, M. (2013). HIV-associated chronic immune activation. *Immunological Reviews*, *254*(1), 78–101. https://doi.org/10.1111/imr.12079

Pandrea, I., Xu, C., Stock, J. L., Frank, D. N., Ma, D., Policicchio, B. B., … Apetrei, C. (2016). Antibiotic and Antiinflammatory Therapy Transiently Reduces Inflammation and Hypercoagulation in Acutely SIV-Infected Pigtailed Macaques. *PLOS Pathogens*, *12*(1), e1005384. https://doi.org/10.1371/journal.ppat.1005384

Papasavvas, E., Azzoni, L., Foulkes, A., Violari, A., Cotton, M. F., Pistilli, M., … Montaner, L. J. (2011). Increased microbial translocation in ≤ 180 days old perinatally human immunodeficiency virus-positive infants as compared with human immunodeficiency virus-exposed uninfected infants of similar age. *The Pediatric Infectious Disease Journal*, *30*(10), 877–882. https://doi.org/10.1097/INF.0b013e31821d141e

Park, M.Y., Kwon, H.J., & Sung, M.K. (2011). Dietary aloin, aloesin, or aloe-gel exerts anti-inflammatory activity in a rat colitis model. *Life Sciences*, *88*(11–12), 486–492. https://doi.org/10.1016/j.lfs.2011.01.010

Petschow, B. W. (2014). Bovine immunoglobulin protein isolates for the nutritional management of enteropathy. *World Journal of Gastroenterology*, *20*(33), 11713. https://doi.org/10.3748/wjg.v20.i33.11713

Philpott, D., Girardin, S., & Sansonetti, P. (2001). Innate immune responses of epithelial cells following infection with bacterial pathogens. *Current Opinion in Immunology*, *13*(4), 410–416. https://doi.org/10.1016/S0952-7915(00)00235-1

Pierce, J. L., Cromwell, G. L., Lindemann, M. D., Russell, L. E., & Weaver, E. M. (2005). Effects of spray-dried animal plasma and immunoglobulins on performance of early weaned pigs. *Journal of Animal Science*, *83*(12), 2876. https://doi.org/10.2527/2005.83122876x

Pilakka-Kanthikeel, S., Huang, S., Fenton, T., Borkowsky, W., Cunningham, C. K., & Pahwa, S. (2012). Increased Gut Microbial Translocation in HIV-infected Children Persists in Virologic Responders and Virologic Failures After Antiretroviral Therapy: *The Pediatric Infectious Disease Journal*, *31*(6), 583–591. https://doi.org/10.1097/INF.0b013e31824da0f5

Pilakka-Kanthikeel, S., Kris, A., Selvaraj, A., Swaminathan, S., & Pahwa, S. (2014). Immune activation is associated with increased gut microbial translocation in treatment-naive, HIV-infected children in a resource-limited setting. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *66*(1), 16–24. https://doi.org/10.1097/QAI.0000000000000096

Plaeger-Marshall, S., Isacescu, V., O'Rourke, S., Bertolli, J., Bryson, Y. J., & Stiehm, E. R. (1994). T cell activation in pediatric AIDS pathogenesis: three-color immunophenotyping. *Clinical Immunology and Immunopathology*, *71*(1), 19–26.

Puthanakit, T., Saksawad, R., Bunupuradah, T., Wittawatmongkol, O., Chuanjaroen, T., Ubolyam, S., … Chokephaibulkit, K. (2012). Prevalence and Risk Factors of Low Bone Mineral Density Among Perinatally HIV-Infected Thai Adolescents Receiving Antiretroviral Therapy: *JAIDS Journal of Acquired Immune Deficiency Syndromes*, *61*(4), 477–483. https://doi.org/10.1097/QAI.0b013e31826ea89b

Rajilić-Stojanović, M. (2013). Function of the microbiota. *Best Practice & Research Clinical Gastroenterology*, *27*(1), 5–16. https://doi.org/10.1016/j.bpg.2013.03.006

Resino, S., Seoane, E., Gutiérrez, M. D. G., León, J. A., & Muñoz-Fernández, M. A. (2006). CD4(+) T-cell immunodeficiency is more dependent on immune activation than viral load in HIV-infected children on highly active antiretroviral therapy. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *42*(3), 269–276. https://doi.org/10.1097/01.qai.0000222287.90201.d7

Reynolds, T., & Dweck, A. C. (1999). Aloe vera leaf gel: a review update. *Journal of Ethnopharmacology*, *68*(1–3), 3–37.

Roider, J. M., Muenchhoff, M., & Goulder, P. J. R. (2016). Immune activation and paediatric HIV-1 disease outcome: *Current Opinion in HIV and AIDS*, *11*(2), 146–155. https://doi.org/10.1097/COH.0000000000000231

Sandler, N. G., & Douek, D. C. (2012). Microbial translocation in HIV infection: causes, consequences and treatment opportunities. *Nature Reviews Microbiology*, *10*(9), 655–666. https://doi.org/10.1038/nrmicro2848

Sandler, N. G., Wand, H., Roque, A., Law, M., Nason, M. C., Nixon, D. E., … for the INSIGHT SMART Study Group. (2011). Plasma Levels of Soluble CD14 Independently Predict Mortality in HIV Infection. *Journal of Infectious Diseases*, *203*(6), 780–790. https://doi.org/10.1093/infdis/jiq118

Sandler, N., Zhang, X., Bosch, R., Funderburg, N., Choi, A., Robinson, J., … et al. (2014). Sevelamer does not decrease Lipopolysaccharide or soluble CD14 levels but decreases soluble tissue factor, low density lopoportein (LDL) cholesteral, and oxidized LDL cholesterol levels in individuals with untreated HIV infection. *The Journal of Infectious Diseases*, *210*(10), 1549–1554.

Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*(3), 317–333. https://doi.org/10.1016/j.intimp.2005.10.005

Serrano-Villar, S., Rojo, D., Martínez-Martínez, M., Deusch, S., Vázquez-Castellanos, J. F., Bargiela, R., … Ferrer, M. (2016). Gut Bacteria Metabolism Impacts Immune Recovery in HIV-infected Individuals. *EBioMedicine*, *8*, 203–216. https://doi.org/10.1016/j.ebiom.2016.04.033

Sharma, B. (2014). Oxidative stress in HIV patients receiving antiretroviral therapy. *Current HIV Research*, *12*(1), 13–21.

Sheikh, S. Z., & Plevy, S. E. (2010). The role of the macrophage in sentinel responses in intestinal immunity: *Current Opinion in Gastroenterology*, *26*(6), 578–582. https://doi.org/10.1097/MOG.0b013e32833d4b71

Siberry, G. (2014). Preventing and Managing HIV Infection in Infants, Children, and Adolescents in the United States. *Pediatrics in Review*, *35*(7).

Sierra-Garcia, G., Castro-Rios, R., Gonzalez-Horta, A., Lara-Arias, J., & Chavez-Montes, A. (2014). Acemannan, an extracted polysaccharide from Aloe vera: A literature reveiw. *Natural Product Communications*, *9*(8), 1217–1221.

Smith, R. L., Boer, R. de, Brul, S., Budovskaya, Y., & Spek, H. van der. (2013). Premature and accelerated aging: HIV or HAART? *Frontiers in Genetics*, *3*. https://doi.org/10.3389/fgene.2012.00328

Smithers, G. W. (2008). Whey and whey proteins—From 'gutter-to-gold.' *International Dairy Journal*, *18*(7), 695–704. https://doi.org/10.1016/j.idairyj.2008.03.008

Sohn, A. H., & Hazra, R. (2013). The changing epidemiology of the global paediatric HIV epidemic: keeping track of perinatally HIV-infected adolescents. *Journal of the International AIDS Society*, *16*(1). https://doi.org/10.7448/IAS.16.1.18555

Song, J.Y., Yang, H.O., Pyo, S.N., Jung, I.S., Yi, S.Y., & Yun, Y.S. (2002). Immunomodulatory activity of protein-bound polysaccharide extracted from Chelidonium majus. *Archives of Pharmacal Research*, *25*(2), 158–164.

Sprong, R. C., Schonewille, A. J., & van der Meer, R. (2010). Dietary cheese whey protein protects rats against mild dextran sulfate sodium–induced colitis: Role of mucin and microbiota. *Journal of Dairy Science*, *93*(4), 1364–1371. https://doi.org/10.3168/jds.2009-2397

Stephensen, C. B., Marquis, G. S., Douglas, S. D., & Wilson, C. M. (2005). Immune activation and oxidative damage in HIV-positive and HIV-negative adolescents. *Journal of Acquired Immune Deficiency Syndromes*, *38*(2), 180–190.

Stiksrud, B., Nowak, P., Nwosu, F. C., Kvale, D., Thalme, A., Sonnerborg, A., … Trøseid, M. (2015). Reduced Levels of D-dimer and Changes in Gut Microbiota Composition After Probiotic Intervention in HIV-Infected Individuals on Stable ART: *JAIDS Journal of Acquired Immune Deficiency Syndromes*, *70*(4), 329–337. https://doi.org/10.1097/QAI.0000000000000784

Strategies for Management of Antiretroviral Therapy (SMART) Study Group, El-Sadr, W., Lundgren, J., Neaton, J., Gordin, F., …..Rappoport, C. (2006), CD4+ count-

guided interruption of antiretroviral treatment. *The New England Journal of Medicine,* 355(22), 2283-2296. DOI. 10.1056/NEJMoa062360

Sudjaritruk, T., & Puthanakit, T. (2015). Adverse bone health among children and adolescents growing up with HIV. *Journal of Virus Eradication*, *1*(3), 159–167.

Suresh, D., Annam, V., Pratibha, K., & Prasad, B. M. (2009). Total antioxidant capacity – a novel early bio-chemical marker of oxidative stress in HIV infected individuals. *Journal of Biomedical Science*, *16*(1), 61. https://doi.org/10.1186/1423-0127-16-61

Taye, B., Shiferaw, S., & Enquselassie, F. (2010). The impact of malnutrition in survival of HIV infected children after initiation of antiretroviral treatment (ART). *Ethiopian Medical Journal*, *48*(1), 1–10.

Tenorio, A. R., Chan, E. S., Bosch, R. J., Macatangay, B. J. C., Read, S. W., Yesmin, S., … Wilson, C. C. (2015). Rifaximin has a Marginal Impact on Microbial Translocation, T-cell Activation and Inflammation in HIV-Positive Immune Non-responders to Antiretroviral Therapy – ACTG A5286. *The Journal of Infectious Diseases*, *211*(5), 780–790. https://doi.org/10.1093/infdis/jiu515

Tobin, N. H., & Aldrovandi, G. M. (2013). Immunology of pediatric HIV infection. *Immunological Reviews*, *254*(1), 143–169. https://doi.org/10.1111/imr.12074

Tsukagoshi, S., Hashimoto, Y., Gufii, G., Kobayashi, H., Nomoto, K., & Orita, K. (1984). Krestin (PSK). *Cancer Treatment Reviews*, *11*(2), 131–155.

Varol, C., Zigmond, E., & Jung, S. (2010). Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria. *Nature Reviews Immunology*, *10*(6), 415–426. https://doi.org/10.1038/nri2778

Veazey, R, & Lackner, A. (2003). The mucosal immune system and HIV-1 infection. *AIDS Reviews*, *5*(4), 245–52.

Wallet, M. A., Rodriguez, C. A., Yin, L., Saporta, S., Chinratanapisit, S., Hou, W., … Goodenow, M. M. (2010). Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T-cell activation following therapy: *AIDS*, *24*(9), 1281–1290. https://doi.org/10.1097/QAD.0b013e328339e228

Willumsen, J. (2011). Nutritional care for HIV-infected children. WHO. Retrieved from www/who.int/elena/titles/bbc/nutrition_hin_children/en/

Xu, X., Xu, P., Ma, C., Tang, J., & Zhang, X. (2013). Gut microbiota, host health, and polysaccharides. *Biotechnology Advances*, *31*(2), 318–337. https://doi.org/10.1016/j.biotechadv.2012.12.009

Xu, Z., & Knight, R. (2015). Dietary effects on human gut microbiome diversity. *British Journal of Nutrition*, *113*(S1), S1–S5. https://doi.org/10.1017/S0007114514004127

Yagi, A., P. Yu, B. (2015). Immune Modulation of Aloe vera: Acemannan and Gut Microbiota Modulator: *Journal of Gastroenterology and Hepatology Research*, *4*(8), 1707–1721. https://doi.org/10.17554/j.issn.2224-3992.2015.04.525

Yami, A., Mamo, Y., & Kebede, S. (2011). Prevalence and predictors of intestinal helminthiasis among school children in Jimma zone; A cross-sectional study. *Ethiop J Health Sci*, *21*(3), 167–174.

Yao, H., Chen, Y., Li, S., Huang, L., Chen, W., Lin, X. (2009), Promotion proliferation effect of a polysaccharide from Aloe barbadensis Miller on human fibroblasts in vitro. *International Journal of Biological Macromolecules,* 45(2), 152-156.doi. 10.1016/j.ijbiomac.2009.04.013

Zeitz, M., Ullrich, R., Schneider, T., Kewenig, S., Hohloch, K., & Riecken, E. O. (1998). HIV/SIV Enteropathy. *Annals of the New York Academy of Sciences*, *859*(1 INTESTINAL PL), 139–148. https://doi.org/10.1111/j.1749-6632.1998.tb11118.x

CHAPTER 2

Methods

Study Design and Participants

A 12-week randomized, blinded interventional trial was conducted with a convenience sample of HIV+ children (tested and confirmed with rapid test serology kits) residing in Addis Ababa, Ethiopia, and enrolled at the All Africa Leprosy and Tuberculosis Rehabilitation and Training (ALERT) Hospital Pediatric HIV Clinic. One hundred and twenty children between the ages of 4-11 years old who had been maintained on antiretroviral therapy (ART) for at least 6 months were enrolled in this trial. The requirement of being well-established on treatment was done to control for the impact of newly initiated treatment on immune parameters, viral loads and anthropometric measures. It is well documented that response to ART, in regards to CD4 recovery and weight gain, is rapid over the first 3-6 months and then tapers off significantly (Asfawesen, Solomie, Bisirat, Mebratu, & Rahlenbeck, 2011; Mulu, Liebert, & Maier, 2014). The ages of 4-11 were selected to incorporate children who could comply with trial requirements for ingesting a drink mix daily. Also, limiting the age to 11 limited the potential impact of puberty on anthropometric and immune measurements (Majaliwa, Mohn, & Chiarelli, 2009).

The trial protocol entitled “Evaluation of Antihelminth Treatment and an Aloe Enriched Whey Protein Drink on Measures of HIV Enteropathy in HIV+ Children in Addis Ababa, Ethiopia”, was approved by the Michigan State University Human Research Protection Program (MSU HRRP IRB# 15-892F/ APP# i047672), the ALERT/AHRI Ethics Review Committee (AAERC No.: PO34/15), the Ethiopian

National Research Ethics Review Committee (ENRERC No. 3.10/134/2016)), and the Ethiopian Food, Medicine, and Healthcare Administration and Control Authority (EFMHACA No 02/6/33/15)m

Sample Size and Groups

Sample size for this trial was estimated using the Java applet for power and sample size (http://www.stat.uiowa.edu/~rlenth/Power), based on an estimated standard deviation of 1.5 within groups and a power of 80% to detect a 30% effect size difference between groups with regard to the primary outcome of plasma intestinal fatty acid binding protein (I-FABP). Based on this calculation, it was determined that 46 children were needed in each group. To account for possible attrition, 60 children were enrolled in each group.

Interventions

Aloe Enriched Whey Protein Drink- Two hundred and fifty mg of aloe vera powder (BiAloe, Lorand Labs, Houton, TX, USA) was added to 2 tablespoons (TBLS) of chocolate-flavored whey protein mix (Combat Powder, MusclePharm, Denver, CO, USA). This serving size is approximately 1/3 of an adult serving and provides approximately 45 calories, 250 mg of aloe, and 8 gm of protein.

Chocolate Drink Mix- As an isocaloric comparison, Nesquik™ chocolate drink powder (Nestlé, Glendale, CA, USA) mixed with nonfat dry milk powder (Mix and Drink, Saco, USA), in a 2:1 ratio was packaged in 2 TBLS servings which provided approximately 45 calories and 0.8 gm protein.

Individual servings were placed into 3x3 zip lock bags and labeled with group number. A week's supply of these baggies was distributed along with a plastic cup with

lid and instructions for mixing at enrollment and at each weekly follow up visit any unused baggies from the week before were collected and a new week's supply given. Parents and guardians were instructed to mix drink powders with 4-8 oz of bottled or boiled water. Plastic cups with lids were provided to allow for thorough mixing and parents were instructed to wash these between uses. New cups were provided each month.

Albendazole- Two 200-mg chewable Albendazole tablets (Eapozole, East African Pharmaceutical Pvt. Ltd. Co., Addis Ababa, Ethiopia) or 20ml Albendazole suspension (Alwo, Leben Laboratories, Pvt, Ltd, Mumbai, India) were provided to all study participants, as well as to family members living in the same household, as a one-time dose to treat intestinal parasites. Dosing is the same for adults and children. This was used to prevent confounding of intestinal parasites on measures of immune stimulation as intestinal parasites are well documented for contributing to immune stimulation (Borkow et al., 2000; Kalinkovich et al., 1999; Walson, Herrin, & John-Stewart, 2009).

* All children were maintained on their established treatment regimens throughout the trial.

Trial Mechanics

Before initiating the trial, clinic nurses, providers and staff were trained by this investigator in trial procedure including enrollment, follow-up visits, and adverse event assessment and reporting, and were acquainted with all data collection and reporting forms. Nursed were also trained in how to do anthropometric measurement accurately and as described in the materials and methods section. A research coordinator from Armauer Hansen Research Institute (AHRI) assisted with the facilitation of this project.

His role was to coordinate and negotiate stipends for clinic nurses, staff and phlebotomists, assist with training of nurses, be available by phone to study participants for adverse event reporting, and to assist in overseeing the trial activities in the clinic.

Enrollment and follow up. At their regularly planned 12-week clinic visit, parents or guardians who were interested and met inclusion/exclusion criteria (Appendix A) were presented with study information including study format, requirements, risks, and benefits and each potential participant was screened against the inclusions/exclusion criteria listed below.

Inclusion Criteria

- Enrolled at the ALERT Hospital Pediatric HIV Clinic for greater than 1 year or with complete records transferred from another Pediatric HIV Clinic.
- Age between 4-11 years
- History of cART for > 6 months.
- Able/willing to return to clinic weekly to collect supplement and complete weekly health history form.
- Able/willing to participate in every 12-week extended clinic visits and blood draws
- Able/willing to ingest study supplements

Exclusion Criteria

- Any physical condition that precludes ingestion of food and study interventions
- Documented coinfection with TB
- Any known cancers
- Hx of hepatic or renal failure
- Neurological/neuromuscular disease (cerebral palsy, muscular dystrophy, etc.)

- Enrollment in another research study or project with medicinal or nutritional interventions
- Children who have been on nutritional therapy (for severe wasting or PEM) in the last 6 months.
- History of non-compliance with therapy
- Having another family member enrolled in this study.

Each parent/guardian received a copy of an information and consent form in Amharic, their local language, and provided signed consent. See Appendices B and C for English version of the Research Participant Information and Consent Form and Human Subjects Consideration and Protections. Nurses read the form to anyone who was not able to read. Since it is not common to inform children of their HIV status in Ethiopia, this trial was described to parents or guardians first without their child in the room and then described to the child in terms of a nutritional trial with no mention of their HIV status.

The enrollment process included an interview, TB screening, a brief physical exam including height, weight and mid upper arm circumference measurements, and phlebotomy. After providing informed consent, participants were randomized to one of two trial groups based on a computer-generated random number list and assigned an identification number:

Group 1: 60 children received an initial dose of Albendazole followed by daily servings of a chocolate flavored, aloe-enriched, whey protein drink

Group 2: 60 children received an initial dose of Albendazole followed by daily servings of an isocaloric chocolate-flavored drink.

See Appendix D for trial case report form (CRF) that was used to capture screening and enrollment screening and. A one-time dose of Albendazole was given to all participants, and additional doses of Albendazole were given for each family member residing in the household to treat endemic intestinal parasites. This was done to address cross contamination between family members from the traditional practice of eating without utensils from a common platter. At the conclusion of their clinic visit, parents or guardians were given instructions for reconstitution of the drink mix, and the first dose of study intervention was ingested by the child. The importance of using clean boiled or bottled water for reconstitution was emphasized as well as the importance of the enrolled child ingesting the full dose daily alongside their daily ART medications. A week's supply of supplement in individual packets was then distributed along with the weekly travel stipend.

Participant's parents or guardians returned to the clinic every week to return any unused supplement packets, receive the following week's supplements, and complete a brief health history form. See Appendix D for Weekly Follow Up Form. At this time a travel stipend and a small prize for the child were also given. Any participants who did not return for their weekly appointment were called and encouraged to return for their visit. In a few instances throughout the trial, and usually with prearrangement, study supplements were distributed for longer time frames to accommodate holidays, planned travel and work schedules of parents or guardians. At the conclusion of 12 weeks, nutritional supplementation was discontinued, and participants returned to the clinic for a final health history and repeat of all measurements including a second collection of blood for assays.

Blinding. During this trial blinding was maintained for all participant families and clinic staff. The site primary investigator was the only unblinded person. Group assignment was designated by numbers only which were present on all daily packets, study forms and participant visit cards.

Adverse event monitoring. During enrollment parents or guardians were instructed to call the research coordinator with any suspected adverse events or to return to the clinic for assessment by a clinic practitioner who completed an adverse event reporting form. See Appendix F for Adverse Event Reporting Form. Severity of adverse events was established using the Common Toxicity Criteria (CTC) scale v4.0, U.S. Department of Health and Human Services, NIH (2009). A data safety and monitoring board (DSMB), consisting of three independent pediatricians was established prior to initiation of the trial. See Appendices G and H for Data Safety and Monitoring Plan and DSMB Charter.

Blood collection and handling. At enrollment and conclusion of 12 weeks, 8-10 ml of blood was collected in EDTA and clot tubes by ALERT Hospital phlebotomists for study assays. After collection, EDTA tubes were centrifuged at 2,000 RPM for 15 minutes. Excess serum and plasma were aliquoted into Eppendorf tubes, labeled by study number and medical record number and frozen at -80C for ELISA assays. All blood was handled using universal precautions as well as protocols for dealing with biohazards as established by AHRI research lab. All assay preps were conducted under a level 2 biosafety hood, and samples decontaminated with bleach and discarded in appropriate sealed containers.

Outcome Measures

The main outcome for this trail was I-FABP. While serum levels of LPS and sCD14 are frequently used correlates of microbial translocation, recently assays for I-FAPB have been incorporated as sensitive markers of enterocyte damage, helping to further clarify causes of immune activation in HIV populations (Hunt et al., 2014; Sandler et al., 2011; Somsouk et al., 2015). Secondary outcomes included determinants of immune activation (sCD14, cellular activation markers CD38 and HLA-DR) which are standard markers in evaluating immune activation. (French et al., 2009; Meditz et al., 2011). Immune reconstitution and treatment response was assessed using standard CD4 counts and viral loads. As incomplete immune reconstitution is a recurrent finding with HIV, the importance of monitoring CD4 T-cell subsets undergoing regeneration has been highlighted as a more sensitive marker of T cell reconstitution (Levy et al., 2009). In light of this and knowing that children reconstitute mainly through thymic emigrants, T-cell subsets to differentiate new thymic emigrants from newly generated peripheral naïve T-cells were assessed by flow cytometry using a repertoire of well-established cell markers (CD31, CD62L, CD45RA, and Ki67) (Bains, Thiébaut, Yates, & Callard, 2009; Kohler & Thiel, 2009; Levy et al., 2009).

Clinical outcome measures included anthropometric measurements (height, weight and mid-upper arm circumference (MUAC)), as well as laboratory measurements of hemoglobin, creatinine, serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT).

Materials and Methods

ELISAs**-**I-FABP and sCD14 were assayed using sandwich ELISA kits according to

manufacturer instructions (Hycult, Netherlands) on plasma stored at -80C. Aliquots of plasma for I-FABP were diluted 2X and for sCD14 320X and assayed in duplicate. See Appendix H for more details.

Cell Staining and Flow Cytometry- To identify surface and intracellular markers of T-cell activation (CD38+HLA-DR+CD4+ and CD38+HLA-DR+CD8+ T-cells) and proliferation (CD31+CD45RA+CD62L+Ki-67+CD4+), cells were stained using fluorochrome conjugated antibodies (CD4-PE, CD8-BV421, CD31-BV510, CD38-PECy7, CD45RA-APC-H7, CD62L-APC, HLA-DR-PerCP5.5 and Ki-67-FITC (BD Biosciences)), according to optimized standard protocols and acquired on an 8 color Canto Flow Cytometer (BD FACSCantoTM II) with Diva software. T-cell subsets were identified and quantified using FlowJo_v9.9.4 software. See APPENDIX H for protocol details.

Anthropometrics- Heights were measured on a platform stadiometer with the child's shoes removed, their back to the height rod and flat headpiece loosely resting on the flat of their heads and measured to the nearest 0.2 centimeter. Weights were measured on a digital scale with shoes and outer clothing removed and measured to the nearest 0.1 kilograms. Mid upper arm circumferences (MUAC) were measured using a MUAC tape on the mid upper arm with the arm hanging loosely by the side and measured to the nearest 0.1 centimeter. Heights and weights were converted into Height for age (HAZ), weight for age (WAZ), and weight for height (WHZ) z-scores and BMI percentages using the Quesgen online pediatric z-score calculator(*www.quesgen.com/BMIPedsCalc.php*) and entered in a REDCap database (https://redcap.bric.msu.edu/).

Laboratory Data-Viral loads were performed on plasma samples by hospital laboratory

technicians using an Abbot n2000sp for extraction and Abbot m2000rt for quantification of copies/ml. CD4 counts, CBCs, hemoglobin and hematocrits as well as liver function panels (SGOT & SGPT) and creatinine determinations were performed by the hospital and AHRI labs.

Data Analysis

All data was imported in JMP v6 software (SAS, Cary, NC). Descriptive statistics were used to report demographic data. Nominal data were reported by percentages and frequencies whereas continuous data described by means, median and interquartile ranges. Comparison of outcome variables was determined using either paired t-tests or ANOVAs for variables with normal distributions and Wilcoxon signed rank test for variables with non-normal distributions. Correlation analyses were done with Pearson's pairwise correlations except for correlations with viral loads which were done using Spearman's rank correlation. Significant differences between groups was determined at the 0.05 level of significance. Each outcome variable was independently assessed. Because we considered this exploratory research, no adjustments were made for multiple comparisons.

APPENDICES

APPENDIX A

Research Participant Information and Consent Form

You are being asked permission for your child to participate in a research study. Researchers are required to provide a consent form to inform you about the research study, to convey that participation is voluntary, to explain risks and benefits of participation, and to empower you to make an informed decision. You should feel free to ask the researchers any questions you may
have.

Study Title: Evaluation of Antihelminth Treatment and an Aloe Enriched Whey Protein Drink on Measures of HIV Enteropathy in HIV+ Children in Addis Ababa

Researchers and Titles: Dr. Rawleigh Howe, MD, PhD; Mary Vander Wal, FNP

Department and Institution: Armauer Hansen Research Institute; CMIB, Michigan State University

Address and Contact Information: Ketema-Lideta, Kebela- 12, Mobile- 0912045608

Sponsor: Armauer Hansen Research Institute

1.PURPOSE OF RESEARCH

You are being asked to allow your child or the child in your care to participate in a research study to evaluate the potential benefit of administering antihelminths (medication for intestinal worms) and 2 nutritional supplements on measures of intestinal health and response to HIV care. Researchers have identified that infection with HIV causes damage to the lining of the intestinal tract and this in turn is felt to have long-term impacts on disease outcomes and response to treatment. The presence of intestinal worms can also have deleterious effects on health and treatment responses in

HIV+ individuals. The proposed research study will explore if administering antihelminths, and nutritional supplements can help heal the mucosal lining of the intestinal tract and if a 12-week period of supplementation can bring about improvements in measures of immune activation and clinical measures such as weight gain.

- Your child has been identified as a possible participant in this study because of their being enrolled at the ALERT Pediatric Clinic, being between the age of 4-11 years old and their current HIV status. All participants in this study need to have been on antiretrovirals for at least 6 months and meet the other inclusion criteria for this study.
- From this study, the researchers hope to learn whether the addition of antihelminths as well as whey protein and an aloe supplement into the diet can improve intestinal health and impact disease outcomes in children with HIV
- Your child's participation in this study will take about 5 minutes each day
- All participants in this study will need parental or guardian permission
- For this study 120 children are being asked to participate.
- This study is being conducted collaboratively with the Armauer Hansen Research Institute

2. WHAT YOU AND YOUR CHILD WILL DO

The staff at the ALERT Pediatric Clinic will continue to provide your child's routine care as always. Your child should continue with their regular prescribed medications and clinic visits. As part of partition in this study the following will be required of you and your child.

- At the time of enrollment into this study all children as well as their whole family units

will be given a suspension or chewable dose of worming medication to treat intestinal worms. The worming medication provided to your child and your family is standard treatment for intestinal worms and is generally well tolerated. It is possible to experience mild stomach upset or cramping as a result of this medication. This alone can have positive impacts on your child's and family's health.

- At the time of enrollment your child's blood will be drawn according to your normal clinic schedule, however a small extra amount may be taken for additional testing. The total amount of blood drawn will be 8-10 ml-approximately 2 teaspoons.
- In addition to your child's current daily medications they will be asked to drink a chocolate flavored drink every day. This will be about ½ cup and will be a dry mixture that you mix in water each day.
- This is a comparison study. There will be 2 groups of patients. Half of the children in the study will drink a plain flavored drink every day and half will drink a flavored drink that contains 2 nutrients (whey protein and aloe) of interest.
- Once a week you will need to return to the clinic for a new supply of supplements and to answer a few questions about the child's health during the past week such as episodes of diarrhea or fever or trips to the clinic. This should be a very brief interaction. If you do not return for your follow up visit a health extension worker may call and/or visit your home to remind you of your clinic appointment
- At your next 12-week clinic visit your child will again have measurements taken and have their blood drawn.
- The participants in the study will be assigned randomly, that is, by a method of chance, to one of the 2 groups. Your child will have an equal chance of being in either

group of the study (e.g. antihelminth + supplement group vs. antihelminth + flavored drink group).

- This study is blinded which means you and your child will not be told which group you are in. The clinic staff will also not be told which group you are in until the end of the study period. All of the data will be entered by study number only into data analysis.
- If you or your child are determined to be non-compliant with study requirements your child will be dropped from the trial.

3. POTENTIAL BENEFITS

The potential benefits to your child for taking part in this study are:

1. The benefit of eradication of intestinal parasites with potential to positively impact health
2. The potential for positive impact on intestinal health from supplementation.
3. Weekly visits to the clinic will allow for closer monitoring and reporting of your child's health status.
4. There is the potential that this research would positively impact the health of not only your child, but the knowledge gained could lead to benefits for many other children with HIV.

4. POTENTIAL RISKS

The potential risks of participating in this study are limited but may include:

1. A potential for an adverse reaction to the worming medication. This medication (Albendazole) is a common medication prescribed by clinics in Ethiopia for intestinal parasites for both adults and children, however the following side effects while not common have been documented: short term abdominal pain, diarrhea, headache,

dizziness, and in very rare cases a temporary elevation of liver enzymes or decreased counts of different blood cells.

2. There are no known significant adverse effects from either of the supplements used in this trial however there is always the chance of an allergic reaction to any substance. An allergic reaction may present with GI disturbance, skin rash or respiratory symptoms.
3. There is the potential that your child will be identified as HIV+ by others attending the clinic during enrollment sessions however measures will be taken to guard your child's diagnosis.
4. There is the potential risk of temporary discomfort from blood draw or the development of phlebitis (irritation of the blood vessel) at site of blood draw
5. There is low potential risk of infection at site of blood draw or contraction of blood infection.

If an adverse reaction or allergic response is suspected or any other concerns arise while participating in this trial, you are encouraged to call the research coordinator Bamlak Tessema at any time of day or night at 0911 419 633

All reported adverse events will be reviewed by the chief investigator (Mary Vander Wal) and Dr. Meseret from the ALERT Pediatric Clinic and referrals made to appropriate physicians or clinics if deemed necessary.

PRIVACY AND CONFIDENTIALITY

-All information and data for this project will be kept confidential.Although we will make every effort to keep your data confidential there are certain times, such as a court order, where we may have to disclose your data.

-Each child that participates in this study will have a folder where all forms and data including lab reports will be kept. These folders will be kept in a locked cabinet that only those directly responsible for this study will have access to. All records will be stored, both electronic and paper, for a minimum of 3 years after trial completion.

-All data collected during this study will be entered into a REDCap online data base by number identifiers only.

-The Intuitional Review Boards from MSU, AHRI, and the Ethiopian National Science and Technology Ethics Review Board will also have access to this data.

-There is a possibility that the U.S. Food and Drug Administration may inspect the records.

-The patient names linked to number identifiers on entered data will be kept on a list locked up with study folders and accessed by the chief investigator or data entry person only.

-The results of this study may be published or presented at professional meetings, but the identities of all research participants will remain anonymous.

7. YOUR RIGHTS TO PARTICIPATE, SAY NO, OR WITHDRAW

Participation of your child in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. You may discontinue participation at any time without penalty or loss of benefits to which you are otherwise entitled including access to care at ALERT Pediatric Clinic.

- You have the right to say no.
- You may change your mind at any time and withdraw your child from this study.
- You may choose not to answer specific questions or to stop participating at any time.
- Choosing not to participate or withdrawing your child from this study will not make any

difference in the quality of any treatment you may receive.

- Whether you choose to participate or not will have no effect on your medical care.
- You will be told of any significant findings that develop during the course of the study that may influence your willingness to continue to have your child participate in the research.
- If you choose to withdraw your child from this study, please notify the research coordinator Bamak Tessema at 0911 419 633.
- If your child is deemed non-compliant with activities outlined for their participation in this study, they may be terminated from this study
- Your child may also be withdrawn from the study if they develop an adverse reaction to study interventions

8. COSTS AND COMPENSATION FOR BEING IN THE STUDY

- Procedures being performed for research purposes only will be provided free of charge
- All antihelminths and supplements for this study will be provided free of charge
- A small stipend of 50 Birr will be provided weekly to cover transportation costs to the clinic for follow up visits.
- No other compensation will be provided to you or your child for participating in this study
- You and your child will not receive money for participating in this study.
- Small items such as crayons, pencils or candy may be given to your child to reward them for their cooperation in this study

9. THE RIGHT TO GET HELP IF YOUR CHILD EXPERIENCES AN ADVERSE EVENT DIRECTLY RELATED TO STUDY PARTICIPATION

If your child experiences an adverse event as a direct result of their participation in this

project, the ALERT Hospital Pediatric Clinic/Hospital will be available for obtaining care. The Armauer Hansen Research Institute will assume responsibility for financial compensation for serious project related adverse events up to 10,000 birr per participant. The lead investigator's institution in the US, Michigan State University, will not provide financial compensation for lost wages, disability, pain or discomfort, unless required by law to do so. This does not mean that you are giving up any legal rights you may have. You may contact *Mary Vander Wal at 0912045608* or the study Research Coordinator Bamlak Tessema at 0911 419 633 with any questions or to report an injury.

10. CONFLICT OF INTEREST

The researchers on this study have no conflicts of interest to report

11. CONTACT INFORMATION

If you have concerns or questions about this study, such as scientific issues, how to do any part of it, or to report an injury, please contact the research coordinator, Bamlak Tessema at 0911 419 633 and he in turn will communicate your concerns to the chief investigator, Mary Vander Wal

- If you have questions or concerns about your role and rights as a research participant, would like to obtain information or offer input, or would like to register a complaint about this study, you may contact, anonymously if you wish, the AHRI/ALERT Ethics Review Committee (0118-962183) or Michigan State University's Human Research Protection Program at 517-355-2180, Fax 517-432-4503, or e-mail irb@msu.edu or regular mail at 408 W. Circle Drive,207 Olds Hall, MSU, East Lansing, MI 48824.

12. DOCUMENTATION OF INFORMED CONSENT

Your signature below means that you voluntarily agree to have your child participate in

this research study.

______________________________ ____________________

Signature Date

APPENDIX B

Human Subjects Consideration and Protections

Informed Consent

- Informed consent was obtained from all parents or guardians of participants at enrollment.
- The informed consent was translated into Amharic and was read to those who are unable to read.
- Enrollment sessions included an overview of the study dynamics, requirements, risks and benefits as well as first measurements of outcome variables and dosing of antihelminths.
- It was not stated in front of children that this was a study evaluating outcomes in HIV patients as most children have not been informed of their diagnosis.

Randomization

- At the time of enrollment participants were randomly placed into intervention groups using a computer-generated number table to either control group or Aloe/Whey group

Blinding

- All participants as well as clinic staff were blinded throughout the study. The site investigator was not blinded. All data was entered into a database by study number only.

Risks

- The treatment for intestinal parasites provides minimal risk for an adverse event. The treatment proposed is standard and routine in this population and the benefit of treatment far outweighs any potential risk. Adverse events most often

associated with the use of antihelminths include abdominal pain, N/V, and diarrhea and these are usually mild and self-limiting.

- The discomfort associated with lab draws. While this procedure is uncomfortable, it is of short duration and the benefits outweigh the risks of discomfort. There is very little risk of significant trauma or health impact from this procedure. Blood for trial assays will be timed with routine clinic labs to avoid extra discomfort and loss of blood. The limit for any one blood draw is set at 10ml to avoid excessive blood loss.
- As with any ingested substance, there is always the risk for allergy to some component in the nutritional supplements.

Benefits

- Children enrolled in the trial as well as their families received free treatment for intestinal parasites.
- Half of the enrolled children also received nutritional supplementation free of charge. Each of these nutritional interventions alone or in combination has the potential to be of benefit on nutritional status.
- Small incentives in the way of reimbursement for transportation costs and small prizes to encourage ongoing participation with study requirements were distributed weekly.
- The knowledge gained from this trial has the potential to positively impact this sample of children as well as many others.

Justice

- All children from the ALERT Pediatric Clinic who met criteria were allowed to

enroll up until the number of enrollees was met.

- All children and their families continued to receive care regardless of their decision to participate in this trial. In addition, dropping out of this trial at any point did not jeopardize their ongoing care.
- In the event of significant adverse events requiring premature discontinuation of the trial all participant families would have been informed

Safety

- No Participant was enrolled without securing his/her guardian's voluntary consent
- All nutritional interventions were provided from the same lot and samples of each will be kept for analysis in the event of an adverse event.
- Clinic nurses were trained in assessment of adverse reactions and documentation/reporting of such incidents. They also were trained regarding supplement distribution, record keeping, and an established clinic referral protocol.
- Trial investigators and/or clinic practitioners were notified of any suspected adverse events and appropriate forms completed.
- A Data Safety Monitoring Board was established to review study data and make determinations regarding early discontinuation of the trial in the event of adverse events. Orientation of all members to the trial protocol and adverse event assessment and reporting was completed prior to enrollment.
- A trial investigator reviewed all laboratory reports generated from the Alert Pediatric Clinic (CD4/CD8 counts, anemia panels and chemistry profiles) for abnormal results before data was entered into the trial database and abnormal

results were brought to the attention of the clinic physician.

- Nutritional status and safety/tolerability of supplementation were assessed by anemia panels and chemistry panels.
- Any child whose lab results are significantly abnormal at enrollment or throughout the trial were referred to the clinic for evaluation and treatment. In addition, any child who is thought to experience a significant adverse response to any intervention throughout the study period was referred to the ALERT clinic physician for evaluation and follow-up.

<u>Security procedures</u>

- The Co-PI maintained records of study group assignments by number. All other individuals involved in study mechanics remained blinded throughout the trial period.
- The data from each participant was kept separate throughout the trial and labeled with study ID only whenever possible. While information on some forms may have the participant's name attached, such as lab results from local clinic lab, data forms will be labeled with number only and will be kept in a locked cabinet.
- The project coordinator will be the only person with access to the master list of participants' names and study numbers and study group assignments. This list will be kept in a locked cabinet.

APPENDIX C

CRF

1. Full Name: (Name –space-father's name)

2. Gender: ☐ 1. Male ☐ 2. Female

3. Age: ☐☐ . ☐☐ yrs. mths (Example 5 yrs 12 weeks = 05.03)

I. Pre Enrollment-Screening for TB and ART

4. Cough of greater than 2 weeks? ☐ 1.Y es ☐ 2. No

5. Wt.loss or poor wt gain? ☐ 1. Yes ☐ 2. No

6. Contact history? ☐ 1. Yes ☐ 2. No

7. Fevers? ☐ 1. Yes ☐ 2. No

8. Ever had TB before? ☐ 1. Yes ☐ 2. No ☐ 3. Unknown

If YES, date of diagnosis (leave blank if unknown): (ddmmyy EC) ___/___/___

If precise date unknown, estimate how long ago: ☐ . ☐☐ (years.months)

(Example: if patient says 1 yr and 10 months, write 1.10)

9. Treatment for TB: ☐ 1. Currently ☐ 2. Completed

10. ART for greater than 6 mths? ☐ 1. Yes ☐ 2. No

Participant ID: ___ ___ ___
Initials: ___________
Visit Date

II. **Enrollment Acceptance Criteria**

<u>Inclusion criteria</u>: (If any marked No, patient is excluded)

	1. Yes	2. No
• Enrolled at the ALERT Hospital Pediatric HIV Clinic for greater than 6 months or with treatment records transferred from another Pediatric HIV Clinic.	☐	☐
• Age between 4-8 years	☐	☐
• History of cART for > 6 months	☐	☐
• Able/willing to return to clinic weekly for trial follow up visits	☐	☐
• Able/willing to participate in an extended clinic visit and blood draws	☐	☐
• Able/willing to ingest study supplements	☐	☐
• Parent/guardian willing to consent to study	☐	☐

<u>Exclusion Criteria</u>: (If any marked YES, patient is excluded)

	1. Yes	2. No
• Any physical condition that precludes ingestion of study interventions	☐	☐
• Suspected to have or with documented co-infection with TB disease	☐	☐
• Any known cancers	☐	☐
• History of hepatic or renal failure	☐	☐
• Neurological/neuromuscular disease	☐	☐
• Enrollment in other medicinal or nutritional Intervention research study	☐	☐
• History of non-compliance with therapy	☐	☐
• Having another family member enrolled in this study	☐	☐
• History of antihelminth treatment in last 12-weeks	☐	☐
• **Eligible for Enrollment**	☐	☐

Participant ID: ___ ___ ___
Initials: ____________
Visit Date

III. Enrollment Demographics

11. DOB: (dd/mm/yyyy) | | | | | | | | |

(If missing day or month, Ethiopian use ‘0’. Eg Nov. 1990 = 00/11/1990)

12. DOB: (dd/mm/yyyy) | | | | | | | | |

(If missing day or month, Gregorian use ‘0’. Eg Nov 1990 = 00/11/1990)

13. Date of enrollment:(ddmmyy)

| | | | | | | | | EC (dd/mm/yyyy)

| | | | | | | | | GC (dd/mm/yyyy)

14. Clinic Card Number: | | | | | |

Address

15. | | | | | | | | | | | | | | | | | | |

Kifle Ketema

16. | | | | | |

Kebele:

17. House Number: | | | | |

18. Mother’s Name:

| | | | | | | | | | | | | | | | | | | | |

19. Tel: | | | | | | | | | | | Mother

20. Father’s Name:

| | | | | | | | | | | | | | | | | | | | |

21. Tel: | | | | | | | | | | | Father

Participant ID: __ __ __
Initials:__________
Visit Date GC:__________

V. Examination and Samples collection

22. Is child acutely ill now or in last 2 weeks

Y______ N______

23. Temp (°C): ☐☐.☐

(If subject temp is 37.0 write 37.0 not 37)

23a. Temp above 37.8 ☐ 1. Yes ☐ 2. No

If yes to 22 or 23a, have patient return in 2 weeks for reassessment and initiation of trial interventions.

24. Date to return. __ __/__ __/__ __ __ __ GC

Reassessment:

Follow up Temp (°C): ☐☐.☐

Anthropometric Measurements: To be completed when child is well

25. Weight: ☐☐.☐ kg

26. Height: ☐☐☐.☐ cm

27. Mid Upper Arm Circumferen ☐☐.☐ cm

(If subject circumference is 26.0 write 26.0 not 26)

Blood Draw:

Serum (1 tube) Tube 1 ☐ 1. Yes ☐ 2. No

Heparin (2 tubes) Tube 1 ☐ 1. Yes ☐ 2. No

Tube 2 ☐ 1. Yes ☐ 2. No

Intervention Distribution:

28. Date interventions initiated: __ __/__ __/__ __ GC

Albendazole administered: ☐ 1. Yes ☐ 2. No

No. of doses given for family: ☐☐

Week supply of supplements given: ☐ 1. Yes ☐ 2. No

Day to return to clinic: ☐ 1. Monday 2. Tuesday

3. Wednesday 4. Thursday 5. Friday

Travel stipend given: ☐ 1. Yes ☐ 2. No

V. Enrollment Status

Patient status: ☐ **1. Enrolled** ☐ **2. Excluded**

29. Informed Consent Signed: ☐ Child assent signed: ☐

30. Date Informed Consent signed:(dd/mm/yy) EC: __ __/__ __/__ __ _ _

GC: __ __/__ __/__ __ _ _

31. Family given copy of information form: ☐

32. Participant ID: ☐☐☐

All pages filled completely: ☐ **1. Yes** ☐ **2. NO**

____

Nurse Signature

IV. HEALTH HISTORY-to be completed by physician

Participant ID: __ __ __
Initials:___________
Visit Date GC:____________

33. Last recorded CD4 count: ☐☐☐☐

34. CD4 count date (GC dd/mm/yyyy):

_____/_____/_______

35. Recent illness (in past 2 weeks): ☐ 1. Yes ☐ 2. No

36. What were/are symptoms of illness:

- ☐ 1. Fever
- ☐ 2. Sore throat
- ☐ 3. Headache
- ☐ 4. Abdominal Pain
- ☐ 5. Nausea/Vomiting
- ☐ 6. Diarrhea
- ☐ 7. Other Specify ______________

37. Date ART started: _____/_____/_______ (EC) (mm/dd/yyyy)

38. Date ART started: _____/_____/_______ (GC) (mm/dd/yyyy)

39. Current ART regimen:
- ☐ 1. -AZT/3TC/NVP
- ☐ 2. -AZT/3TC/EFV
- ☐ 3. -AZT/3TC/KALETRA
- ☐ 4. -ABC/3TC/KALETRA
- ☐ 5. – Other

  5a. If other, please specify ______________________

40. Recent medication other than ART: ☐ 1. Yes ☐ 2. No

Participant ID: ___ ___ ___
Initials: ____________
Visit Date GC: _____________

39. Name of the medication if known:

- ☐ 1. Amoxacillin
- ☐ 2. Amoxacillin/clavulonate (Augmentin)
- ☐ 3. Ceftrixone
- ☐ 4. Ciprofloxacin
- ☐ 5. Cloxacillin
- ☐ 6. Cotrimoxazole (theraprim)
- ☐ 7. Ketoconazole (Dezor, shampoo)
- ☐ 8. Metronidazole
- ☐ 9. Miconazole
- ☐ 10. Paracetamol
- ☐ 11. Prednisone or equivalent
- ☐ 12. Other ______________________

40. Current WHO stage: ☐ (1,2,3, or 4)

41. If excluded reason for exclusion: __________________________

42. Referral made: ☐ 1. Yes ☐ 2. No

43. Further investigations: ☐ Yes ☐ 2. No

If yes, specify: ___________________________________

44. Treatment: ☐ 1. Yes ☐ 2. No

If yes, specify: ___________________________

Participant ID: ___ ___ ___
Initials: ____________
Visit Date GC:

VII. Laboratory Results- Initial at Enrollment

45. Height for age Z score + ☐ - ☐ 45a. ☐.☐☐

46. Weight for age Z score + ☐ - ☐ 46a. ☐.☐☐

47. Weight for Height (BMI): ☐☐.☐

48. MUAC: ☐☐.☐ cm

49. CD4 Ct: ☐☐☐☐ cubic mm

50. CD8 Ct: ☐☐☐☐ cubic mm

51. CD4/CD8 Ratio: ☐.☐

52. Viral Load: ☐☐☐☐☐☐☐ **always place leading zeros!!

56. HGB: ☐☐.☐ gm/dL

** If below 10 place a 0 in first box

53. Hct % ☐☐.☐

54. AST: ☐☐☐.☐ units/liter

55. ALT: ☐☐☐☐.☐ units/liter

56. Creatinine: ☐.☐ mg/dL

Participant ID: ___ ___ ___
Initials: ____________
Visit Date GC:

12 Week Follow Up

Visit Date________________________GC

57. Height for age Z score + ☐ -☐ ☐.☐☐
58. Weight for age Z score + ☐ -☐ ☐.☐☐
59. Weight for Height (BMI): ☐☐.☐☐
60. MUAC: ☐☐.☐☐ cm
61. CD4 Ct: ☐☐☐☐ cubic mm
62. CD8 Ct: ☐☐☐☐ cubic mm
63. CD4/CD8 Ratio: ☐.☐
64. Viral Load: ☐☐☐☐☐☐☐ **always place leading zeros!!
65. HGB: ☐☐.☐gm/dL ** If below 10 place a 0 in first box
66. Hct % ☐☐.☐ %
67. AST: ☐☐☐.☐ units/liter
68. ALT: ☐☐☐.☐ units/liter
69. Creatinine: ☐.☐ mg/dL

APPENDIX D

Weekly Follow Up Form

Name________________________ Study ID # ___________________

Date _______________ Week # _____________

Health History Questions:

- In the past week has your child:
  1. Visited the clinic

     Yes _______ No________

     Number of visits ___________
  2. Been prescribed medication

     Yes ________ No________

     Type/Name of medication (if known) ________________
  3. Had a fever

     Yes _______ No_______
  4. Had any episodes of diarrhea (stool that isn't formed)

     Yes _______ No _______ If so how many days with diarrhea ______________
  5. New skin rash

     Yes _______ No _______

Left over amount ______________ sachets from previous week

Parent or guardian comments (other symptoms experienced):

____

____

If child is currently experiencing any symptoms listed in questions nos. 1-5 refer child to Dr. Meseret or clinic physician for evaluation of adverse reaction

New week's supply of supplement given?_______

Travel stipend given?_______

Pt evaluated by clinic physician and adverse event screening form completed:

____

Signature of nurse

APPENDIX E

Suspected Adverse Event Reporting Form

Project Title: Evaluation of Antihelminth Treatment and an Aloe Enriched Whey Protein Drink on Measures of HIV Enteropathy in HIV+ Children in Addis Ababa, Ethiopia

Today's Date: ____________________________

IRB#_______________________

Participant Name__________________________________

Study #____________________

Gender: M_____ F______ Age____________ Group

#_____________

Length of time in study in weeks/days (i.e. 2 weeks and 3 days)

____

Problem/AE Onset Date: / /

Problem/AE Termination Date: / / ____ N/A____ Event

Continuing

1. Provide a detailed description of the problem/adverse event including date, time, location, duration, etc.

2. Action taken in response to Problem/AE:

3. Relevant tests (e.g. x-rays) and results: ____ N/A

4. Describe treatment(s) of Problem/AE (Include medications used to treat this event.): ____ N/A

5. List name of Concomitant Medications (Do not include medications used to treat this event.)

____ N/A

6. Describe pre-existing conditions/relevant clinical history: ____ N/A

7. Outcome of the Problem/AE:____ Recovered/resolved
____ Recovering/resolving
____ Not recovered/not resolved
____ Recovered/resolved with sequelae
____ Fatal
____ Unknown
____ Other ______________________________

Seriousness of the Problem/AE (check all that apply):	____ Non-serious ____ Emotional/Psychological Harm ____ Required medical intervention ____ Required hospitalization ____ Life-threatening ____ Death If death, date of death: / / ____ Other
Severity of the Problem/AE	____ Mild ____ Moderate ____ Severe ____ Life-Threatening ____ Fatal ____N/A
Problem/AE Attributed to:	____ Unknown ____ Study medication ____ Study supplement ____ Underlying disease ____ Errors in study medication administration, or deviations ____ Breach of Confidentiality ____ Other suspected cause (describe on separate sheet) ____ Research Subject Complaint (describe on separate sheet)
Has the same Problem/AE occurred previously in this study?	____ Yes ____ No If yes, how many times?

____ Unanticipated ____ Anticipated ____In Assessor's judgment is prompt reporting needed

In Assessor's judgement is problem/AE related to study intervention Y_____ N______

Should child be dropped from the trial Y_____ N________?

Signature____________________________________
Reporting Timeframe:
All problems/adverse events that are severe or life-threatening, AND unanticipated AND which are related to the study procedures must be reported to the IRB.
1.Any anticipated or unanticipated death of a subject occurring that is related to the study procedures should be reported immediately (i.e. within 48 hours).
2.A problem/adverse event experienced by a subject that is life threatening and unanticipated, and related to the study procedures, should be reported within 7 calendar days (1 week) of investigator's receipt of information.
3.All other severe and unanticipated problems/adverse events that are related to study procedures, must be reported within 14 calendar days (2 weeks) of investigator's receipt of information

APPENDIX F

Data Safety and Monitoring Plan

Evaluation of Antihelminth Treatment and an Aloe Enriched Whey Protein Drink on Measures of HIV Enteropathy in HIV+ Children in Addis Ababa, Ethiopia

PI – Rawleigh Howe, MD, PhD (rawcraig@yahoo.com)

Co-PI- Mary Vander Wal, MSN, RN, FNP (vande521@msu.edu)

Co-Investigators:
Barbara A. Smith, PhD, RN, FACSM, FAAN (Barbara.Smith@hc.msu.edu)
Meseret Gebre, MD (mesibire@yahoo.com)

Research Coordinator: Bamlak Tesema

Brief Description of Study:

It is now known that an early and ongoing impact of HIV infection is damage to the intestinal mucosa. This allows translocation of bacterial components into the general circulation which initiates a state of chronic immune activation which in turn impacts disease progression and contributes to the development of multiple co-morbidities. Damage to the intestinal mucosa also impacts absorption of nutrients and contributes to compromised growth in children. Antiretrovirals are not effective at eradicating viral reserves in the intestinal tract and therefore the pathology in the gut is ongoing. Nutritional supplements incorporating amino acids and/or aloe polysaccharides have been documented to support tissue healing and attenuate release of pro-inflammatory cytokines in animal models of colitis and it is hypothesized that their daily ingestion will positively impact measures of mucosal integrity, bacterial translocation, immune activation, oxidative stress and clinical outcomes in HIV+ children. A 12-week randomized clinical trial with 120 HIV+ children ages 4-8 who have been on antiretrovirals for greater than 6 months will be used to explore these interventions.

Albendazole will be administered to all children at enrollment to address confounding of abdominal parasite infections which can also damage the intestinal mucosal. Study participants will be divided into 2 groups. The intervention group will ingest daily 12 grams of a chocolate flavored whey protein drink that is enriched with 250mg of aloe whole leaf powder. A comparison group will ingest daily an isocaloric equivalent chocolate flavored drink. A weekly supply of individual daily sachets will be distributed by clinic nurses and a brief health history and screening tool completed each week. Other study outcome data will be collected at the conclusion of the 12-week intervention period. Paired T tests and correlation methods will be used to analyze study data.

Risk Assessment:

This trial is anticipated to provide low risk of adverse events

Plan for Monitoring and Safety Review:

- All participants will be screened for inclusion/exclusion criteria prior to enrollment by clinic nurses and Mary Vander Wal and any questionable participants will be reviewed by Dr. Meseret.
- Adherence to treatment and potential adverse events will be monitored during weekly visits where unused sachets of trial interventions will be collected, and Weekly Follow Up Forms completed.
- Any participant parent who does not return to the clinic weekly will be followed up by Health Extension Workers (CHEW) who will make a home visit.
- If a participant is determined to be non-compliant with study interventions for greater than 2 weeks they will be dropped from the trial.
- A data safety and monitoring board (DSMB) will be convened and will be

responsible for monitoring potential adverse events and making a determination on early closure of the study in the event of a significant number of adverse events the safety environment of participants.

Plan for Adverse Event Reporting:

ALERT Pediatric Clinic Nurses will be conducting interviews on a weekly basis to track participants' health history and screen for potential adverse reactions (See Weekly Follow Up Form). In the event that a participant develops significant symptoms during off hours of the clinic, parents will be directed to contact the research coordinator. (This person will be appointed by the Armauer Hansen Research Institute but has yet to be determined)

Any child with reportable illness or potential adverse reaction will be evaluated by Dr. Meseret Gebre or designated clinic physician in her absence within 48 hrs. and a Potential Adverse Event Form completed. Dr. Meseret can be contacted at *mesibire@yahoo.com*. Dr. Meseret is a co-investigator on this project, however she will not be responsible for day to day operations of this trial other than confirming eligibility in questionable situations and evaluating children who are reported to have potential adverse reactions. Since she may see children enrolled in this trial if they present in the clinic for another purpose she will remain blinded to intervention group status of participants. This will help to protect blinding of enrolled children.

If a child is reported to have symptoms that may indicate an adverse event an Adverse Event Reporting form will be completed and forwarded to the chair of the DMSB. IF the child's symptoms are significant enough to drop them from the study, the chair will consult with the primary investigators and the research coordinator and if warranted the

group status of the participant will be revealed.

The potential risks of participating in this study are expected to be limited but may include:

1. A potential for an adverse reaction to the worming medication. This medication (Albendazole) is a common medication prescribed by clinics in Ethiopia for intestinal parasites for both adults and children, however the following side effects while not common have been documented: short term abdominal pain, diarrhea, headache, dizziness, and in very rare cases a temporary elevation of liver enzymes or decreased counts of different blood cells.
2. There are no known significant adverse effects from either of the supplements used in this trial however there is always the chance of an allergic reaction to any substance. An allergic reaction may present as a GI disturbance, skin rash, or respiratory symptoms.
3. There is the potential that a child will be identified as HIV+ by others attending the clinic during enrollment sessions however measures will be taken to guard your child's diagnosis.
4. There is the potential risk of temporary discomfort from blood draw or the development of phlebitis (irritation of the blood vessel) at site of blood draw
5. There is low potential risk of infection at site of blood draw or contraction of blood infection.

- The co-primary investigator, Mary Vander Wal, will review all data collected weekly and will discuss any findings from an adverse event evaluation with Dr. Meseret or other clinic physician.

- The Common Toxicity Criteria (CTC) scale will be used to grade the severity of adverse events.
- Any severe adverse events will be reported to the research coordinator who will hold the list of group assignment. The blinding may be broken in individual cases to assist in determining if the adverse event was linked to the study intervention.
- A severe adverse event will result in the participant being dropped from the trial, treated by the clinic if warranted and monitored on an ongoing basis until resolution of symptoms.
- Minor and moderate symptoms will be monitored but will not result in breaking blinding unless they are persistent (beyond 1 week) or recurrent (reported more than twice in weekly screenings).
- If 10% or greater of the children from the intervention group experience significant adverse events the study will be stopped prematurely, and all IRBs notified of such.
- It will be the PI's responsibility to inform MSU, AHRI, and the Ethiopian IRBs if the investigator terminates or suspends the clinical trial.

Plan for Data Management:

The research coordinator from AHRI and secondary investigator (Mary Vander Wal) will be responsible for collection and storage of data. All data collected in paper form will be stored in individual participant files that will be kept in a locked cabinet. All data will be entered into a computerized spreadsheet by study number only and backed up after each entry. Paper copies of data will be stored through the duration of the trial and beyond.

Human Subject Training:

All co-investigators on this trial have completed the required Human Subjects Training

APPENDIX G

DSMB Charter

AWPT: Aloe and Whey Protein Trial

Independent Data Safety and Monitoring Board Charter

Prepared by: Mary Vander Wal

Co-Principle Investigators-Dr. Rawleigh Howe and Mary Vander Wal

February 2016

Content Guidance	Charter Details
Introduction	
Name (& Sponsor ID) of trial	Evaluation of Antihelminth Treatment and an Aloe Enriched Whey Protein Drink on Measures of HIV Enteropathy in HIV+ Children in Addis Ababa, Ethiopia Funded by AHRI and supplemental grant funds
Ethical Review	Michigan State University IRB ALERT/AHRI IRB Ethiopian National Research Ethics Review Committee Food, Medicine and Healthcare Administration and Control Authority, Addis Ababa
Trial Objectives, including interventions being investigated	**Background:** It is now known that an early and ongoing impact of HIV infection is damage to the intestinal mucosa. This allows translocation of bacterial components into the general circulation which initiates a state of chronic immune

activation which in turn impacts disease progression and contributes to the development of multiple co-morbidities. Damage to the intestinal mucosa also impacts absorption of nutrients and contributes to compromised growth in children. Antiretrovirals are not effective at eradicating viral reserves in the intestinal tract and therefore the pathology in the gut is ongoing. Nutritional supplements incorporating amino acids and/or aloe polysaccharides have been documented to support tissue healing and attenuate release of pro-inflammatory cytokines in animal models of colitis and it is hypothesized that their daily ingestion will positively impact measures of mucosal integrity, bacterial translocation, immune activation, oxidative stress and clinical outcomes in HIV+ children. A 12-week randomized clinical trial with 120 HIV+ children ages 4-8 who have been on antiretrovirals for greater than 6 months will be used to explore these interventions. Albendazole will be administered to all children at enrollment to address confounding of abdominal parasite infections which can also damage the intestinal mucosal. Study participants will be divided into 2 groups. Study outcome data will be collected at enrollment and at the conclusion of the 12-week intervention period. Paired T tests and correlation methods will be used to analyze study data.

	Aim: To evaluate outcomes of treatment for intestinal parasites and nutritional interventions on measures of HIV enteropathy **Design*:*** Prospective, randomized blinded trial **Outcome measures:** Clinical outcome measures- Anthropometric measurements, Measures of bacterial translocation: sCD14, I-FABP, endotoxin **Population:** 120 HIV+ children ages 4-8 who have been on antiretrovirals for greater than 6 months will be used to explore these interventions **Groups:** After trial invitation and consent for inclusion, a random number table will be used to decide which group the child will receive be allocated to. The intervention group will ingest daily 12 grams of a chocolate flavored whey protein drink that is enriched with 250mg of aloe whole leaf powder. A comparison group will ingest daily an isocaloric equivalent chocolate flavored drink. A weekly supply of individual daily sachets will be distributed by clinic nurses and a brief health history and screening tool completed each week.
Outline of scope of Charter	The purpose of this document is to describe the membership, terms of reference, roles and responsibilities of the

	independent DSMB for this trial.

Roles and Responsibilities	
Aims of Committee	To safeguard the interests of trial participants, monitor the main outcome measures including safety and efficacy, monitor adverse event occurrence and monitor the overall trial conduct
Terms of reference	The DSMB should receive information on enrollment and adverse events and provide advice on the conduct of the trial to the Principal Investigators Dr Rawleigh Howe and Mary Vander Wal.
Specific roles of DSMB	Interim review of the trial's progress including updated data on recruitment, data quality, protocol violation, follow-up. Specifically: Monitor evidence for differences in the outcome measures by the two arms Monitor harms – Numbers of reported potential adverse events and especially serious adverse events Decide whether the trial follow-up should be stopped earlier Assess data quality and completeness Maintain confidentiality of all trial information that is not in the public domain

	Monitor recruitment figures and losses to follow-up Monitor protocol compliance by participators and investigators Suggest additional data analysis as necessary
Before or early in the trial	
DSMB assumptions	All DSMB members have seen the protocol before agreeing to join the committee All relevant ethical clearance has been successfully approved before recruitment starts Reservations by DSMB members about the trial should be raised with the Principal Investigators. DSMB members should be supportive of the aims and methods but remain independent and constructively critical of the ongoing trial.
Timing of the 1st meeting	Summer 2016, before the trial starts recruiting the committee will meet in open session to discuss the practicalities as necessary.
DSMB contract	DSMB members should formally register their ascent to join the group by signing Annex A indicating that they agree with the contents of this charter. All members of the DSMB will submit CVs to the ALERT/AHRI IRB
Composition	
Members of the	Dr Solomie Derebessa (Pediatrician and HIV specialist,

DSMB	Clinton Foundation). Dr. Kullehe Haddis (Pediatrician, Girum General Hospital) Dr Million Shibeshi (Pediatrician Save the Children)
DSMB chairman	The board will identify a chairman who will provide written feedback to the co-PIs in a timely manner after committee meetings
Relationships	
Relationships with PI other trial investigators, sponsors and regulatory bodies	The DSMB is independent of all other regulatory bodies, and of the trial personnel If required, reports from the data monitoring committee will be sent to the ethics committees or other regulators authorities
Payments to DSMB members	Members will be reimbursed 1000 birr each for committee meetings attended. No other payments or rewards are provided
DSMB conflict of interest	Competing interests should be disclosed. These are not restricted to financial matters but could include involvement in other trials or intellectual investment in the research area.
Organization of meetings	The DSMB will meet prior to recruitment and on an as need basis throughout the trial. Meetings will be SKYPE or face-to-face in Addis Ababa

	A mixture of open or closed sessions may be held. Closed sessions will only be attended by DSMB members, may invite any expert in the field to join them. Open sessions may be joined by the Principal Investigator and any trial member, at the Principal Investigators' discretion.
Trial documentation and procedures to ensure confidentiality and proper communication	
Content of material available during open sessions	Recruitment and data quality – protocol treatment compliance will be presented Total numbers of adverse events will be presented, graded according to the Common Toxicity Criteria (CTC) scale (but not be summarized by treatment group)
Content of material available in closed sessions	In addition to the material available in open session, the closed session material will include assessment of serious adverse events based on participant data by treatment group. Blinding will be broken on an individual basis to assist the DSMB in making determinations to terminate the study
Preparation and use of the reports	Materials for the meetings will be distributed by the AHRI research coordinator or the co-PI Mary Vander Wal prior to each meeting.

	Material showing accumulating data and analysis by randomized group will be available only to the DSMB members and will be kept confidential. DSMB do not have the right to share confidential information with anyone outside the DSMB, including the Principal Investigators.
Responsibility for disseminating external evidence	Identification and circulation of external evidence (e.g. from other trials or from systematic reviews) are not the responsibility of the DSMB members. They may bring in such information should they come across it. The Principal Investigators will collate any such data and present it in open session.
To whom does the DSMB report?	The DMSP reports recommendations to the Principal Investigators The DSMB should destroy their report after each meeting DSMB members should store any of their own notes and papers safely to ensure information about the trial outcomes by treatment arm does not inadvertently come to the attention of trial personnel.

Decision making	
What decisions or recommendations	Possible recommendations: No actions continue as planned

will be open to the data monitoring committee	Early recommendation to stop – clear benefit, harm or external evidence Comments upon Statistical analysis Extending the trial from a pilot phase
How decisions will e reached by the DSMB	The chair is to summarize discussions and encourage consensus; it is usually best for them to give their own opinion last. Every effort should be made for the DSMB to make a unanimous decision. If this is not possible a vote may be taken. These details should not be in the report. It is important that the implications (ethical, statistical, practical, financial) be considered before recommendations are made.
Quorate for decision making	The DSMB will be quorate providing all 3 members are present.
Absent DSMB members	If the data report is circulated before the meeting, DSMB members who will not be able to attend the meeting may pass on comments to the DSMB Chair for consideration.

Reporting	
Who will receive the report?	DSMB reports to the principle investigators within 2 days in the event of a reportable adverse event. The report should not

	contain information about the trial arms. Detailed minutes are not needed. A summary of the main points is sufficient. The person taking the minutes will be agreed before the start of the meeting. The DSMB Chair may sign off any minutes or notes.
After the trial	
Publication of results	DSMB may meet following the trial to discuss final data DSMB members may be given the opportunity to read and comment on publications before submission DSMB members will be named in any report, unless they request otherwise DSMB members should refrain from discussing confidential issues from the closed meetings.

Agreement and potential competing Interests (Annex A)

Aloe and Whey Protein Trial.

Insert your initials ……. below and please return to the Principal Investigators:

…………agree and understand the DSMB charter version 1.1 dated 10/5/15

…………agree to join the Independent Data Monitoring Committee for this trial

…………agree to treat all sensitive trial data and discussions confidentially

Delete as appropriate:

I have no competing interests to declare

I have competing interests to declare

……………………………………………………………………………………

Name:

Electronic Signed:

Date:

Potential competing interests:

- Stock ownership in competing companies
- Frequent speaking engagements on behalf of the intervention
- Career tied up with a product or technique of the trial
- Involvement in the trial running
- Emotional involvement of the trial
- Intellectual conflict e.g. strong prior belief in the trial's experimental arm
- Involvement in regulatory issues relevant to the trials procedures
- Investment or career interest in competing products
- Involvement in the publication, in the form of authorship

APPENDIX H

Details of Lab Assay Protocols

Eight to ten millimeters of blood was collected from each patient and divided between 2 EDTA and 1 clot tubes. Whole blood collected in EDTA tubes was used for CBCs and CD4 counts. The second EDTA tube along with the clot tube were centrifuged at 2,000 RPM for 15 minutes in a Beckman Alegra 5 centrifuge and plasma utilized for viral loads as well as for ELISAs. Serum was used for creatinine, SGOT and SGPT assays. Remaining plasma and serum were aliquoted into 1.5ml Eppendorf tubes, labeled by study number and medical record, and stored at –80°C until use. Blood cells from EDTA collection tubes were resuspended in room temperature phosphate buffered saline (PBS) in an amount proportional to plasma aliquoted and was used in further cell staining experiments.

Assessment of plasma biomarkers of intestinal barrier function- Intestinal Fatty Acid Binding Protein (I-FABP) and Soluble CD14 (sCD14) using ELISA

Both I-FABP and sCD14 were quantified using ELISA kits according to manufacturer directions (Hycult-Biotech, Netherlands). Briefly, all samples and reagents were brought to room temperature and plasma aliquots were diluted 2X for I-FABP assays and 320X for sCD14 assays. One hundred microliters of standards and samples were pipetted in duplicate into 96 well plates that were pre-coated with antibody and incubated covered at room temperature (RT) for 60 minutes. This was followed by a wash procedure that was repeated 3 times. Following washing, 100μl of a biotinylated detection antibody specific for a second site on the mediator was added to all wells using an 8-tip multichannel pipettor and plates were again covered and incubated at RT for 60

minutes. After an additional washing, 100 µl of streptavidin–alkaline phosphatase was added to all wells and the plate was again covered and incubated for 60 minutes. After another washing, 100 µl of the Tetramethylbenzidine (TMB) substrate was added, the plates were covered with aluminum foil and incubated at room temperature for 30 minutes followed by the addition of 100 µl stop solution. The reaction product was measured colorimetrically using a Multiskan EX Primary EIA plate reader with a 450 OD filter and Ascent v2.6 software. The amount of I-FABP and sCD14 was calculated from a standard curve and multiplied by the dilution factor.

3. Immune activation and cell proliferation were assessed using 8 color cell staining and flow cytometry. Immune activation was defined by the counts and percentages of CD38+HLA-DR+ CD8+ and CD38+HLA-DR+ CD4+ T-cells. De novo T-cell proliferation was quantified using CD4+CD45RA+CD62L+CD31+ markers. Homeostatic T-cell proliferation was quantified following surface and intracellular staining for CD4+CD45RA+CD62L+Ki67+ markers.

Assessment of lymphocyte subsets

Separated blood cells from EDTA centrifuged tubes were reconstituted in appropriate amounts of phosphate buffered saline (PBS) and stained for surface and intracellular markers using fluorochrome conjugated antibodies against CD4-PE, CD8-BV421, CD31-BV510, CD38-PECy7, CD45RA-APC-H7, CD62L-APC, HLA-DR-PerCP5.5 and Ki-67-FITC (BD Biosciences) according to the protocol outlined below. Three hundred microliters of cells mixed in PBS was aliquoted into polystyrene flow cytometry tubes followed by a cocktail of optimized amounts of antibody stains (2.5-20µl) and incubated at room temperature for 20 minutes. This was followed by the

addition of 2ml of FACS lysis buffer (BD Biosciences, Catalog No.349202) for an additional 10 minutes at room temperature. The sample was then washed with 2ml FACS buffer (PBA + 1mg/ml serum bovine albumin and 1 mM EDTA) and centrifuged at 1700 RPMs x 5 minutes in a Beckman Alegra 5 Centrifuge. Following the wash, the supernatant was decanted, cells were resuspended and incubated with 500 µl of permeabilizing FACS solution (Perm-2, BD Biosciences, Catalog No.40973) at room temperature for 20 minutes. This was followed by another wash cycle with 3.5 ml FACS buffer followed by centrifuging at 2100 RPM for 12 minutes. After decanting and resuspension 2.5µl of anti-Ki-67 was added and incubated for an additional 20 minutes at room temperature followed by another wash with 4 ml FACs buffer and centrifuged at 2100 RPM x 12 minutes. Cells were then resuspended and fixed with 300 µl of 2% formaldehyde x 30 minutes followed by a final wash with FACS buffer. The stained and fixed cells were then stored at 4C in the dark until acquired into a FACS Canto 8 Color Flow Cytometer (BD FACSCantoTM II) according to manufacturer instructions using compensation microbeads from BD and Diva software. A minimum of 100,000 events per sample were collected for analysis. Lymphocytes were identified based on forward and side scatter (FSC-SSC). Frequencies and percentages of T-cell subsets were calculated from total counts of “parent” populations and were identified and quantified by gating strategies using FlowJo_v9.9.6 software.

REFERENCES

REFERENCES

Asfawesen, G., Solomie, D., Bisirat, T., Mebratu, B., & Rahlenbeck, S. (2011). Outcome in a pediatric cohort receiving ART in Addis Abeba, Ethiopia. *Acta Paediatrica*, *100*(8), 1164–7.

Bains, I., Thiébaut, R., Yates, A. J., & Callard, R. (2009). Quantifying thymic export: combining models of naive T cell proliferation and TCR excision circle dynamics gives an explicit measure of thymic output. *Journal of Immunology (Baltimore, Md.: 1950)*, *183*(7), 4329–4336. https://doi.org/10.4049/jimmunol.0900743

Borkow, G., Leng, Q., Weisman, Z., Stein, M., Galai, N., Kalinkovich, A., & Bentwich, Z. (2000). Chronic immune activation associated with intestinal helminth infections results in impaired signal transduction and anergy. *Journal of Clinical Investigation*, *106*(8), 1053.

French, M. A., King, M. S., Tschampa, J. M., da Silva, B. A., & Landay, A. L. (2009). Serum Immune Activation Markers Are Persistently Increased in Patients with HIV Infection after 6 Years of Antiretroviral Therapy despite Suppression of Viral Replication and Reconstitution of CD4 $^{+}$ T Cells. *The Journal of Infectious Diseases*, *200*(8), 1212–1215. https://doi.org/10.1086/605890

Hunt, P. W., Sinclair, E., Rodriguez, B., Shive, C., Clagett, B., Funderburg, N., … Lederman, M. M. (2014). Gut Epithelial Barrier Dysfunction and Innate Immune Activation Predict Mortality in Treated HIV Infection. *Journal of Infectious Diseases*, *210*(8), 1228–1238. https://doi.org/10.1093/infdis/jiu238

Kalinkovich, A. & Weisman, Z & Greenberg, Z & Nahmias, J & Eitan, S & Stein, M. & Bentwich, Zvi. (1999). Decreased CD4 and increased CD8 counts with T cell activation is associated with chronic helminth infection. *Clinical and experimental immunology*. 114. 414-21. 10.1046/j.1365-2249.1998.00736.x

Kohler, S., & Thiel, A. (2009). Life after the thymus: CD31+ and CD31- human naive CD4+ T-cell subsets. *Blood*, *113*(4), 769–774. https://doi.org/10.1182/blood-2008-02-139154

Levy, Y., Lacabaratz, C., Weiss, L., Viard, J.-P., Goujard, C., Lelièvre, J.-D., … Delfraissy, J.-F. (2009). Enhanced T cell recovery in HIV-1–infected adults through IL-7 treatment. *Journal of Clinical Investigation*, *119*(4), 997–1007. https://doi.org/10.1172/JCI38052

Majaliwa, E. S., Mohn, A., & Chiarelli, F. (2009). Growth and puberty in children with HIV infection. *Journal of Endocrinological Investigation*, *32*(1), 85–90. https://doi.org/10.1007/BF03345686

Meditz, A. L., Haas, M. K., Folkvord, J. M., Melander, K., Young, R., McCarter, M., … Connick, E. (2011). HLA-DR+ CD38+ CD4+ T Lymphocytes Have Elevated CCR5 Expression and Produce the Majority of R5-Tropic HIV-1 RNA In Vivo. *Journal of Virology*, *85*(19), 10189–10200. https://doi.org/10.1128/JVI.02529-10

Mulu, A., Liebert, U. G., & Maier, M. (2014). Virological efficacy and immunological recovery among Ethiopian HIV-1 infected adults and children. *BMC Infectious Diseases*, *14*(1). https://doi.org/10.1186/1471-2334-14-28

Sandler, N. G., Wand, H., Roque, A., Law, M., Nason, M. C., Nixon, D. E., … for the INSIGHT SMART Study Group. (2011). Plasma Levels of Soluble CD14 Independently Predict Mortality in HIV Infection. *Journal of Infectious Diseases*, *203*(6), 780–790. https://doi.org/10.1093/infdis/jiq118

Somsouk, M., Estes, J. D., Deleage, C., Dunham, R. M., Albright, R., Inadomi, J. M., … Hunt, P. W. (2015). Gut epithelial barrier and systemic inflammation during chronic HIV infection: *AIDS*, *29*(1), 43–51. https://doi.org/10.1097/QAD.0000000000000511

Walson, J. L., Herrin, B. R., & John-Stewart, G. (2009). Deworming helminth co-infected individuals for delaying HIV disease progression. In The Cochrane Collaboration (Ed.), *Cochrane Database of Systematic Reviews*. Chichester, UK: John Wiley & Sons, Ltd. Retrieved from http://doi.wiley.com/10.1002/14651858.CD006419.pub3

CHAPTER 3
A Cross Sectional Analysis of Microbial Translocation, Immune Activation and Health Status in a Cohort of HIV+ Children in Addis Ababa, Ethiopia

Abstract

There is increasing concerns about the premature development of co-morbid disease in both HIV+ children and adults, however, few studies have evaluated markers that may indicate this in children, especially in developing countries. We quantified measures of microbial translocation and immune activation and assessed associations with viral loads, anthropometrics and immune status in one hundred and twenty HIV+ children ages 4-11 in Addis Ababa, Ethiopia. Approximately 40% of children demonstrated height for weight (HAZ) or weight for age (WAZ) scores below -2 and 29.7% of children had viral loads > 1,000 copies/ml. Neither HAZ nor WAZ correlated with CD4 or viral load however associations were noted between both measures of growth status and higher viral loads in the distribution of activated and memory T-cell subsets. Growth failure and viremia have significant negative impacts which may contribute to the premature development of co-morbid disease in HIV+ children. This warrants close monitoring and further research.

Introduction

There are an estimated 2.1 million children living with HIV worldwide (WHO, 2016), the vast majority of whom reside in resource-poor countries. Over the 30+ years since the beginning of the AIDS epidemic, survival rates have improved dramatically, and life expectancy has been extended by decades. In spite of this, life expectancy for those infected with HIV is often cut short by several years with mortality in HIV+ adults now being attributed to non-HIV co-morbidities rather than to the viral infection itself

(Bhatia, Ryscavage, & Taiwo, 2012; Deeks, 2011; Franceschi & Campisi, 2014; Kuller et al., 2008; Smith, Boer, Brul, Budovskaya, & Spek, 2013). Immune activation is a hallmark of HIV disease and has been linked to the various co-morbid diseases that are widely documented in adult HIV populations including cardiovascular disease, diabetes, renal disease, cancers, neurocognitive decline and osteoporosis (Appay & Sauce, 2008; Duprez et al., 2012; Hunt et al., 2014;). Adult HIV cohorts display higher markers of chronic immune activation and inflammation than healthy controls and develop age-related co-morbidities at earlier ages (Bhatia et al., 2012; Deeks, 2011; Hunt et al., 2014; Smith et al., 2013). A major contributor to chronic immune activation in HIV populations is microbial translocation which ensues as a result of damage to the mucosal lining of the small intestines early in the disease process (Sandler & Douek, 2012).

It is likely HIV+ children who acquired the infection early in life, will develop co-morbid diseases even earlier than individuals who acquired the infection as adults, however little research has been conducted in pediatric HIV cohorts, especially in developing countries. Little is known about levels of chronic immune activation in pediatric HIV cohorts and verification of appropriate markers for use in pediatric populations is needed.

In this cross-sectional analysis we quantified and did correlations analysis with measures of I-FABP, sCD14, CD4 counts and viral loads as well as anthropometric measurements and T-cell subsets. In doing this our intent was to assess levels of microbial translocation and associations with markers of treatment response and the distribution of T-cells including activation and proliferation subsets in a pediatric HIV

cohort in Ethiopia.

Subjects and Methods

Subjects. Children between the ages of four and eleven, with documented HIV, who had been maintained on ART for a minimum of six months, were recruited between January and July 2017 from the pediatric out-patient clinic at Alert Hospital, Addis Ababa, Ethiopia. The data presented here is from baseline determinations across groups collected as part of a randomized, blinded clinical trial exploring nutritional interventions for HIV microbial translocation and immune activation. At enrollment, participants were screened for known co-morbidities including tuberculosis, hepatitis, liver disease, neurologic disease and cancers. The parents or guardians of all children provided written informed consent. The study proposal was approved by the Human Research Protection Program, Michigan State University (HRPP-MSU), as well as by the Alert/AHRI Ethics Review Committee (AAERC), the Ethiopian National Research Ethics Review Committee (ENRERC), and the Ethiopian Food, Medicine and Health Administration and Control Authority (EFMHACA).

Anthropometric measurements. At enrollment, all children were assessed for height, weight and mid-upper arm circumference (MUAC). Heights were measured on a platform stadiometer with the child's shoes removed, their back to the height rod and flat headpiece loosely resting on the flat of their head and measurements recorded to the nearest 0.2 centimeter. Weights were measured on a digital scale with shoes and outer clothing removed and measured to the nearest 0.1 kilograms. Heights and weights were converted into height for age (HAZ), weight for age (WAZ), using the Quesgen online pediatric z-score calculator (*www.quesgen.com/BMIPedsCalc.php*) according to WHO-

child growth parameters. A z-score of 0 represents the median based on the WHO age/sex reference data. A z-score of -1 represents a measurement that is 1 standard deviation below the reference population. To determine if there is a slowing of growth as HIV children age we further stratified our cohort into 2 groups by age such that ages 8 and under = 0 and ages over 8 = 1 and did further growth evaluation.

Study data were collected and managed using REDCap electronic data capture tools hosted at Michigan State University. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies (Harris et al., 2009).

Clinical laboratory tests. At enrollment, 8-10 ml of blood was drawn from each participant and collected in BD EDTA and clot Vacutainer tubes. Viral loads as well as CD4 counts and complete blood counts (CBC) were performed by hospital laboratory technicians at Alert Hospital. Viral loads were assayed using an Abbot n2000sp for extraction and Abbot m2000rt for quantification of viral copies/ml. CD4 counts were done using a BD FACSounter, while CBCs were run using a Sysmek Kx-21. Remaining plasma and serum was aliquoted into 1.5ml Eppendorf tubes and stored at -80C for ELISAs and further assays.

Assessment of lymphocyte subsets. Immune reconstitution as evaluated based on CD4 counts as well evaluating proliferating subsets using Ki-67, an intracellular marker of homeostatic proliferation. Cellular immune activation was quantified based on T-cell phenotype and expression of surface markers HLA-DR and CD38 on both CD4 and CD8 T-cells. Separated blood cells from EDTA centrifuged tubes were reconstituted in appropriate amounts of phosphate buffered saline (PBS)

and stained for surface and intracellular markers using fluorochrome conjugated antibodies against CD4-PE, CD8-BV421, CD31-BV510, CD38-PECy7, CD45RA-APC-H7, CD62L-APC, HLA-DR-PerCP5.5 and Ki-67-FITC (BD Biosciences) according to the protocol outlined below. Three hundred microliters of cells mixed in PBS was aliquoted into polystyrene flow cytometry tubes followed by a cocktail of optimized amounts of antibody stains (2.5-20µl) and incubated at room temperature for 20 minutes. This was followed by the addition of 2ml of FACS lysis buffer (BD Biosciences, Catalog No.349202) for an additional 10 minutes at room temperature. The sample was then washed with 2ml FACS buffer (PBA + 1mg/ml serum bovine albumin and 1 mM EDTA) and centrifuged at 1700 RPMs x 5 minutes in a Beckman Alegra 5 Centrifuge. Following the wash, the supernatant was decanted, cells were resuspended and incubated with 500 µl of permeabilizing FACS solution (Perm-2, BD Biosciences, Catalog No.40973) at room temperature for 20 minutes. This was followed by another wash cycle with 3.5 ml FACS buffer followed by centrifuging at 2100 RPM for 12 minutes. After decanting and resuspension 2.5µl of anti-Ki-67 was added and incubated for an additional 20 minutes at room temperature followed by another wash with 4 ml FACs buffer and centrifuged at 2100 RPM x 12 minutes. Cells were then resuspended and fixed with 300 µl of 2% formaldehyde x 30 minutes followed by a final wash with FACS buffer. The stained and fixed cells were then stored at 4C in the dark until acquired into a FACS Canto 8 Color Flow Cytometer (BD FACSCantoTM II) according to manufacturer instructions using compensation microbeads from BD and Diva software. A minimum of 100,000 events per sample were collected for analysis. Lymphocytes were identified based on forward and side scatter (FSC-SSC). Frequencies and percentages of T-cell subsets were

calculated from total counts of “parent” populations and were identified and quantified by gating strategies using FlowJo_v9.9.6 software.

Assessment of intestinal barrier. Microbial translocation was evaluated using two surrogate markers namely, intestinal fatty acid binding protein (I-FABP), an intracellular protein released from damaged enterocytes that line the intestinal mucosal, and soluble CD14 (sCD14), a surface acute phase protein released by monocytes upon activation by lipopolysaccharide (LPS) and other antigenic stimuli. The measurement of sCD14 has become an established indicator of microbial translocation and innate immune activation in HIV research involving both adults and children. Both I-FABP and sCD14 were quantified using ELISA kits according to manufacturer directions (Hycult-Biotech, Netherlands). Plasma aliquots were diluted 2X for I-FABP assays and 320X for sCD14 assays.

Statistical analysis. All data was analyzed using JMP software v6 (SAS, Cary, NC). Descriptive statistics were used to report demographic and clinical data. Nominal data were reported by percentages and frequencies whereas continuous data described by means, standard deviation, median and interquartile ranges. Comparison of outcome variables was determined using either t-tests for variables with normal distributions and Wilcoxon test for variables with non-normal distributions. Correlation analyses were done using Pearson’s correlations statistic for parametric and Spearman’s statistic for non-parametric data. Significant differences between groups was determined at the 0.05 level of significance. Some continuous variables, such as HAZ, WAZ and viral load were additionally categorized into high and low values and used as nominal variables to graphically stratify other variables of interest such as T cell subsets, which were then

compared by standard t-tests. (** in general concordance was observed of a given test of significance for correlation of two continuous variables, with a given t-test for the same two variables, one converted to a nominal variable. **) Each outcome variable was independently assessed. Because we considered this exploratory research, no corrections were made for multiple comparisons.

Results

Participant characteristics. Table. 1 provides demographic information for the 120 participants enrolled in this study. There were slightly more males than females enrolled (68 v s 52) and mean age in our cohort was 8.1 years ± 0.2. Growth failure is a common complication of pediatric HIV and can be evidenced by underweight, stunting or both. Our cohort displayed growth failure as evidenced by a mean WAZ score of -1.6, mean HAZ score of -1.6 and mean BMI of 15. Forty percent of our cohort was determined to be undernourished as defined by WAZ or HAZ of <-2 with a total of 31.7% who were underweight and 32.5% who were stunted. Mean duration on ART was 5.1 ± 2.5 years and while there was a variety of regimens, all children in our cohort were on highly active antiretroviral therapy (HAART). Eighty percent of the participants were on a regimen of Zidovudine (AZT), Lamivudine (3TC) and either Nevirapine (NVP) or Efavirenz (EFV), with 10% on AZT, 3TC and Kaletra. The remainder had either Abacavir or Stavudine in replace of AZT, together with 3TC and either NVP or EFZ.

CD4 count and viral load. Due to more active thymic function in children versus adults, children usually have enhanced potential to restore CD4 counts after the initiation of ART (Tobin & Aldrovandi, 2013). Children are also reported to more often demonstrate sustained viremia, even in the presence of good CD4 T-cell reconstitution

following initiation of ART (Rainwaterr-Lovett et al, 2014, Tobin & Aldrovandi, 2013). Mean CD4 count in our cohort was 955 ± 436 cells/mm^3. Mean viral load was 8791 ± 2973 copies/ml, however the distribution was quite skewed with a median of 0 and range of 0 to 170355.

Variable	Mean (Std Dev)	Median (Range)
Number recruited (n=120)		
Sex (male, female)		
Male (n=68)		
Female (n=52)		
Age	8.1 (1.9)	8.1 (4.0-11.2)
Weight for Age Z-score	-1.64 (1.08)	-1.57 (-6.71,0.60)
Height for Age Z-score	-1.61 (1.04)	-1.63 (-4.09,1.63)
Weight for Height Z-score	-0.77 (1.01)	-0.71 (-3.73,1.86)
BMI	15.0 (1.9)	14.8 (1.3,23.1)
Mid Upper Arm	16.44 (1.42)	16.3 (12.1,20.9)
Time on ART(yr)	5.1 (2.5)	5.6 (0.6,9.8)
CD4 cells/mm3	939 (440)	881 (39,2655)
Viral Load (copies/ml) (	8791 (2973)	0 (0,170355)

Table 1. Demographics of participants *Based on number with reported viral load

Subjects were stratified into 4 categories based on viral load, as depicted in Figure 1. 70.3% of the subjects had undetectable viral load, 12.6% had viral loads greater than 10,000 copies/ml, and 6.3% and 10.8% with values from 150-1000 and 1000-10000 copies/ml, respectively. Viral load in our cohort was inversely correlated with CD4 counts ($r = -0.4$, $p < 0.0001$). No correlation was found between CD4 count and WAZ ($r = -.03$, $p = 0.721$) or HAZ ($r = .04$, $p = 0.651$). Similarly, there was no correlation between viral load and WAZ ($r = -.08$, $p = 0.395$) or HAZ ($r = -.13$, $p = 0.155$).

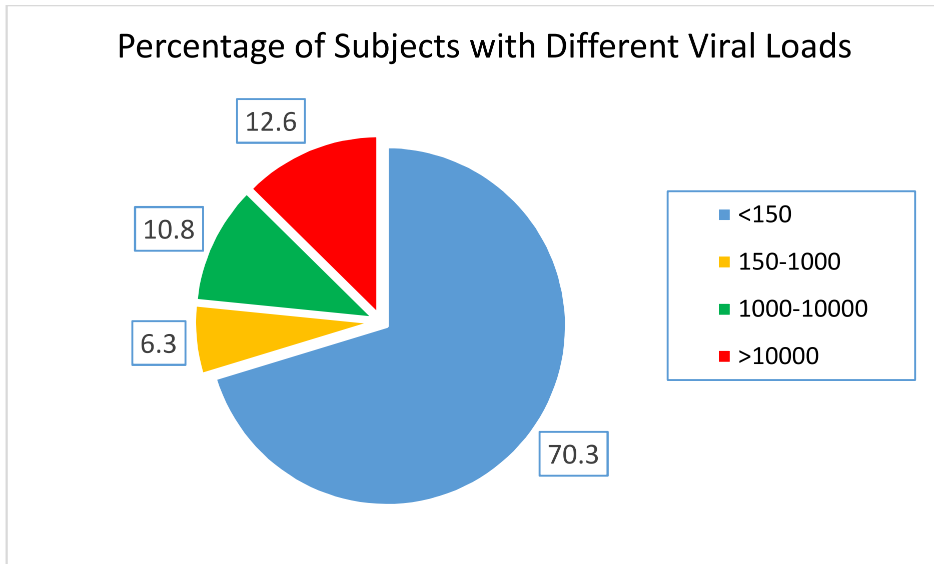

Figure 1. Distribution of viral loads by percentages (N=111)

CD4 and CD8 T cell subsets. While distribution of T cell subsets has been explored thoroughly in adult HIV, fewer studies have been performed in HIV+ children, especially in sub-Saharan Africa. Similar to HIV+ adults, children have been reported to have lower naïve CD4 T cell numbers, higher percentages of memory CD4 T-cells and higher percentages of CD8 effector memory T cells (Rainwater-Lovett et al., 2014; Ssewanyana et al., 2009). Since we were exploring immune activation in conjunction with microbial translocation we were interested to identify T-cell subsets that were expressing the activation markers HLA-DR and/or CD38. Cellular activation is a precursor of proliferation and we were interested to evaluate associations between both activated and proliferating subsets with other study variables. We used the intracellular protein Ki-67 as a marker of proliferation. We used flow cytometry to identify and quantify T-cell subsets in our cohort of children. Figure 2 provides a pictorial of the gating strategy used.

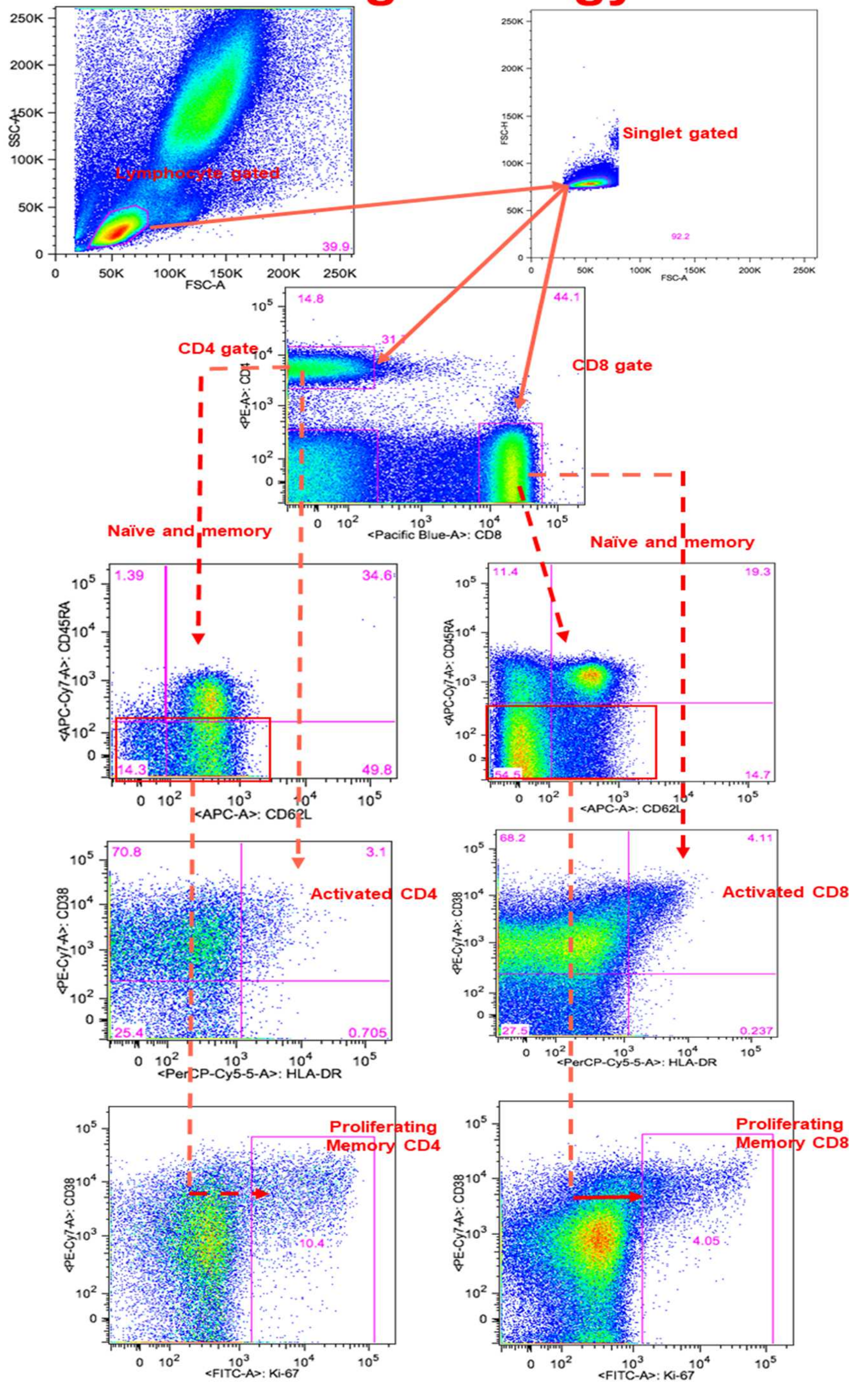

Figure 2. Gating Stragety to identify T cell subsets. Lymphocytes identified based on forward and perpendicular light scatter. After exclusion of cell doublets, CD4 and CD8 subsets were independently gated, and further defined either on the basis of CD45RA and CD62L expression (to further delineate cell differentiation) or CD38 and HLA-DR expression to define activated (CD38+HLA-DR+) cells. Proliferating, Ki-67+ cells were delineated after gating on CD4+ or CD8+ CD45RA- memory cells.

For the purposes of this study, naïve subsets were defined as CD45RA+CD62L+, central memory (CM) T-cells were defined as CD45RA-CD62L+, effector memory (EM) T-cells were defined as CD45RA-CD62L-, and terminally differentiated effector memory (TDEM) T-cells as CD45RA+CD62L-. Activated CD4 and CD8 T-cells were defined as HLA-DR+CD38+ and proliferating memory T-cells were defined as CD45RA- cells which excluded the TDEM (CD45RA+ CD62L-) subset. This subset, while maintaining cytotoxic potential and cytokine production has been shown to have little proliferative capacity which supported our excluding them when quantifying proliferating T cells (Mahnke, Brodie, Sallusto, Roederer, & Lugli, 2013).

Mean percentages of CD4 and CD8 T-cells were 32.2 and 32.7 respectively. The mean naïve CD4 subset was 47.7% while the central memory subset was 36.8%. Effector memory CD4 T-cells comprised 13.8%, terminally differentiated effector memory 1.6%, activated CD4 T-cells expressing both CD38 and HLA-DR totaled 1.8%, and proliferating Ki-67+ memory CD4 T cells 5.1%. Among CD8 T cells, the naïve subset consisted of 30.6%, central memory 13.5%, effector memory 38.8%, and terminally differentiated effector memory 17.0%. CD38+DR+ activated cells, and Ki-67+ proliferating memory cells represented 3.1% and 3.7%, respectively, among CD8 T cells.

The correlation coefficient between CD4 count and naïve CD4 T cells was 0.52 ($p < 0.001$). Conversely, central and effector memory CD4 T cells were negatively correlated ($r = -0.47$, $p < 0.001$; and $r = -0.39$, $p < 0.001$, respectively), whereas terminally differentiated effector memory CD4 showed no correlation with CD4 count ($r = -.03$, p not significant). Similarly, naïve CD8 T cells were positively correlated with

CD4 count ($r = .52$, $p < 0.001$), central memory CD8 T cells showed no correlation ($r = .02$, p not significant), and both effector memory ($r = -.23$, $P < 0.05$) and terminally differentiated effector memory ($r = -0.39$, $p < 0.001$) exhibited strong inverse associations with CD4 count. Activated CD38+HLA-DR+ CD4 T cells ($r = -.33$, $p < 0.001$) and CD38+HLA-DR+ CD8 T cells ($r = -.30$, $p < 0.001$) showed highly significant inverse correlations with CD4 count. Similarly, proliferating memory Ki-67+ CD4 T cells ($r = -.39$, $p < 0.001$) and Ki-67 memory CD8 T cells ($r = -.15$) showed inverse associations with CD4 count, but only the former reached statistical significance. Thus, in general, for both CD4 and CD8 T cells, naïve subsets were positively correlated with CD4 count, and memory subsets inversely correlated with CD4 count. Activated and proliferating CD4 and CD8 T cells were generally inversely correlated with CD4 count.

While neither WAZ nor HAZ showed any correlation with CD4 count or viral load, we noted significant associations with T cell differentiation, but not T cell activation and proliferation. Figure 3 summarizes the distribution of naïve and memory subsets of CD4 and CD8 T-cell subset frequencies among patients stratified into high and low HAZ and WAZ categories. Low WAZ and HAZ were defined as those with z scores less than -1.6, and high as those greater than or equal to -1.6. A general pattern emerged whereby subjects with higher weight or height had relatively high frequencies of naïve CD4 and CD8 T cells, but progressively lower frequencies of memory cells at more differentiated effector stages. However, only four such associations reached statistical significance: the association between HAZ and TDEM CD4 T cells, the association between WAZ and HAZ with naïve CD8 T cells, and the association between HAZ and EM CD8 T cells.

In contrast, no association was observed between HAZ and CD4 CD38+HLA-DR+ CD4 cells (r = -.15, p = 0.096), proliferating CD4 Ki67 memory cells (r = -.06, p = 0.486), CD38+HLA-DR+ CD8 T cells (r = - .09, p = 0.358, or CD8 Ki67+ memory cells (r = -.02, p = 0.820). Similarly, no correlation between WAZ and CD38+HLA-DR+ CD4 T cells (r = .01, p = 0.922), Ki67 CD4 memory T cells (r = .07, p = 0.426), CD38+HLA-DR+ CD8 T cells (r = .00, p = 0.967), or CD8 Ki67+ memory cells (r = .08, p = 0.381) was observed. Thus, the associations were limited to the distribution of naïve and memory T cell subsets, and not apparent for T cell activation, T cell proliferation, CD4 counts or viral load.

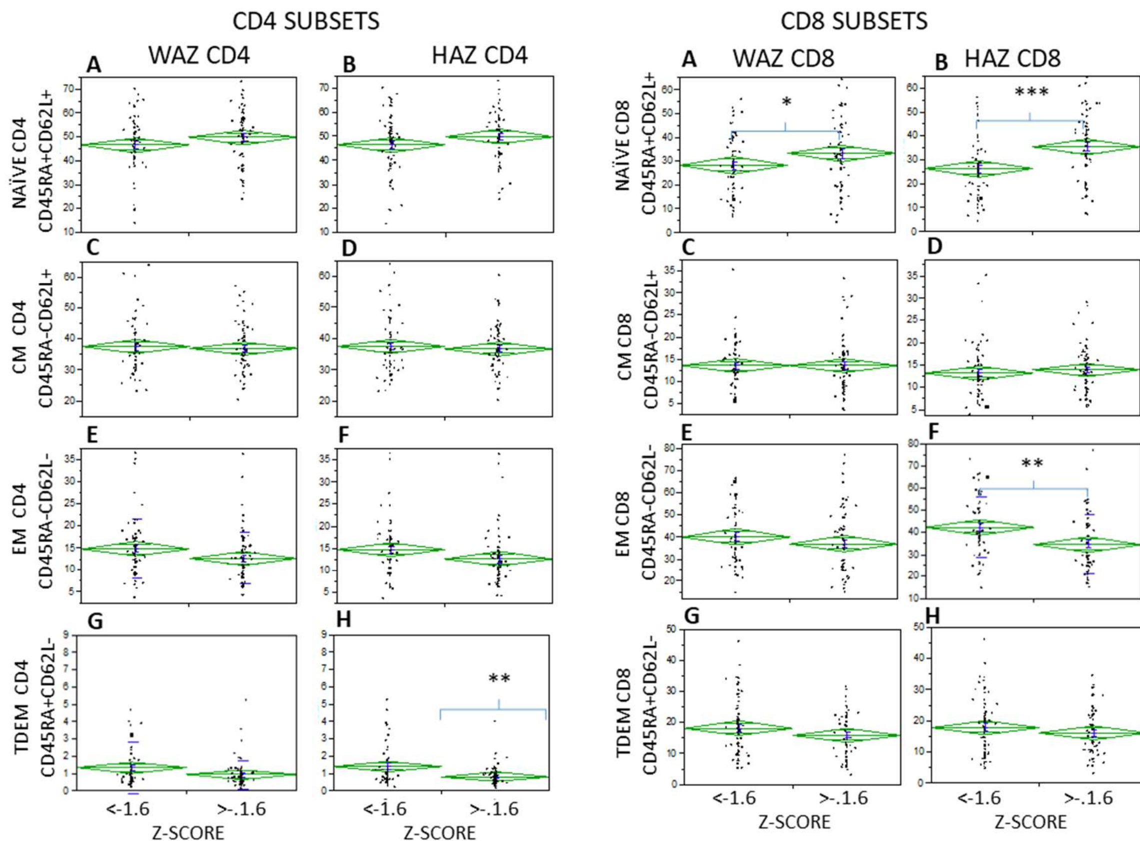

Figure 3. Distribution of CD4 and CD8 subsets by WAZ/HAZ categories
(*- p = 0.05, ** = p <0.01, *** = p <0.001.) A,B = Naïve subsets, C,D = Central Memory subsets, E,F = Effector Memory subsets, G,H = Terminally Differentiated Effector Memory subsets.

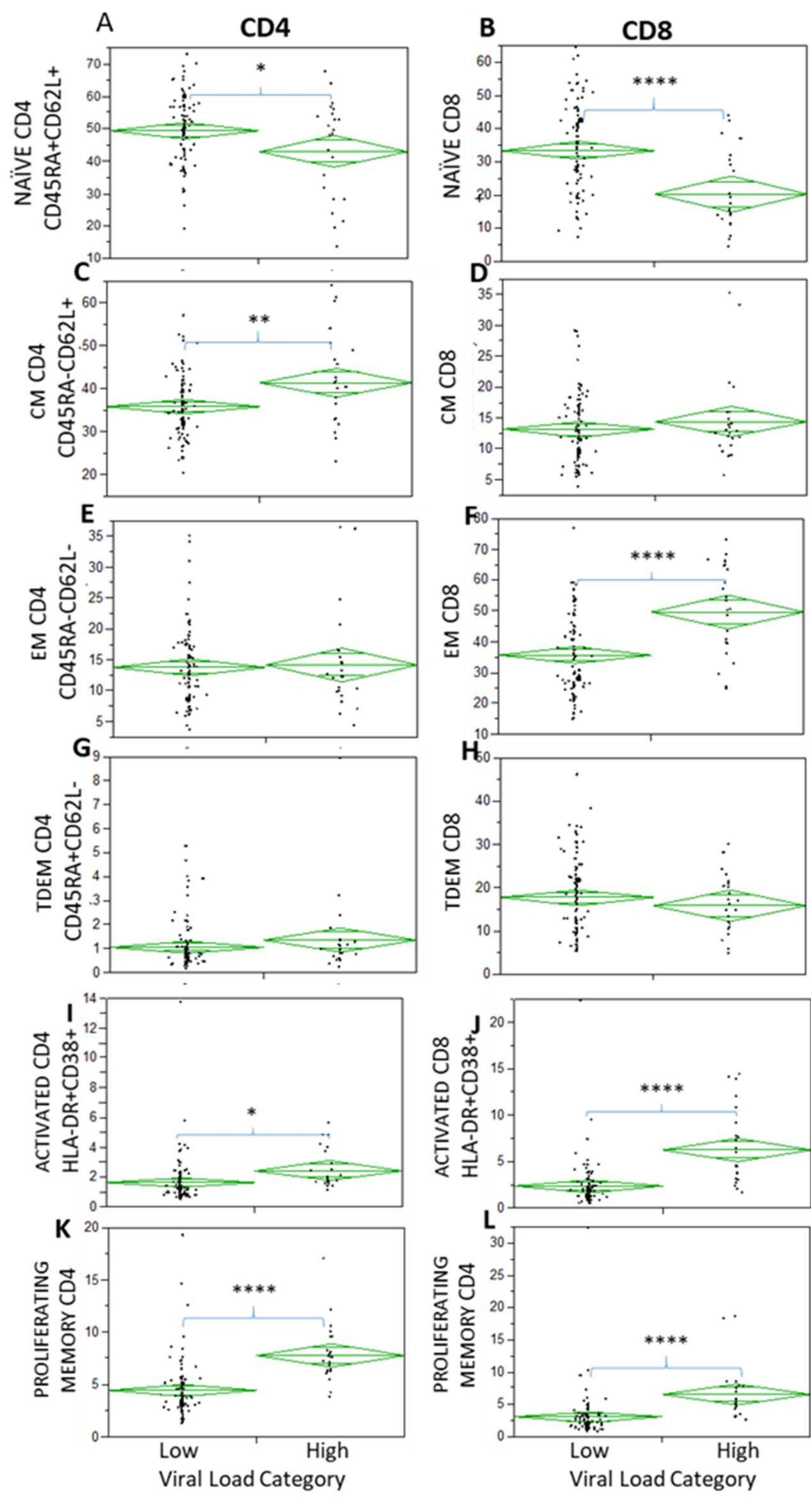

Figure 4. Percent CD4 and CD8 naïve and memory subsets compared by viral load category. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$, A,B -Naïve CD4 and CD8 Subsets, C,D -Central Memory subsets, E,F -Effector Memory subsets, G,H -Terminally Differentiated subsets, I,J - Activated subsets, K,L -Proliferating Memory subsets

Viral load and T cell subsets. As shown in Figure 4, we noted in the high viral load group decreased percentages of naïve T-cells in both CD4 and CD8 subsets ($p = 0.022$ and $p < 0.0001$ respectively). Within the high viral load group percentages of central memory and effector memory subsets differed between the CD4 and CD8 T-cell compartments with an elevated percentage of central memory in the CD4 subset ($p = 0.004$), and an elevated percentage of effector memory in the CD8 T-cell subsets ($p < 0.0001$). The high viral load group also showed increased percentages of proliferating memory and activated cells in both the CD4 ($p \leq 0.000$ and $p = 0.03$ respectively) and CD8 T-cell ($p \leq 0.0001$ and $p \leq 0.0001$ respectively) subsets reaching high statistical significance at each.

I-FABP and sCD14. Several adult HIV studies have utilized I-FABP and sCD14 as surrogates for microbial translocations, and many have reported correlations with markers of immune activation (Brenchley et al., 2006; Hunt et al., 2014; Somsouk et al., 2015). Fewer studies have assessed microbial translocation in pediatric HIV, most of these have been in infants, and most in developed countries. The mean I-FABP in our pediatric cohort was 563+/- 383 pg/ml and mean level of sCD14 was 2879 +/- 1350 ng/ml

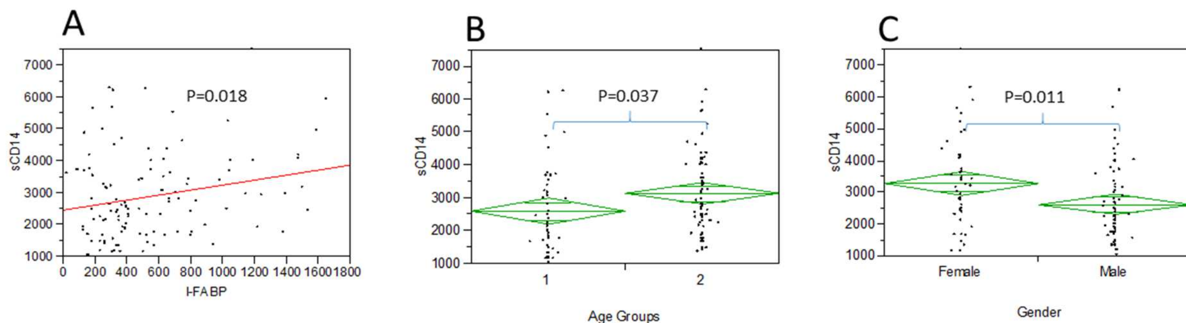

Figure 5. Associations of sCD14 with I-FABP, age, and gender. A. sCD14 vs I-FABP, B. sCD14 vs age (Age <8 =1, Age 8-11 = 2), C. sCD14 levels by gender

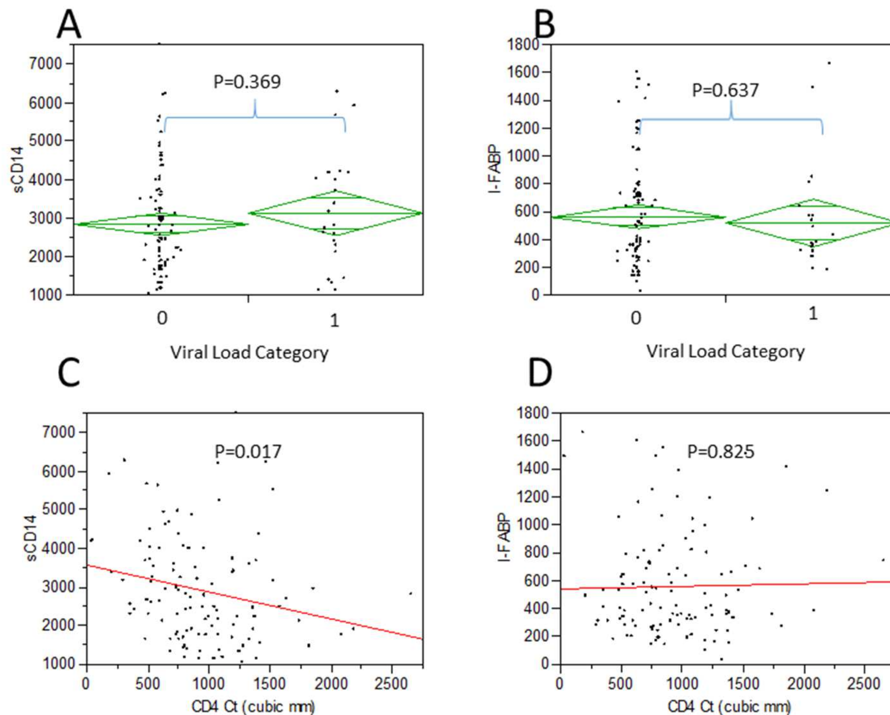

Figure 6. Associations of sCD14 and I-FABP with viral load categories and CD4 count. A. sCD14 by viral categories (<1000copies/ml = 1, >1000 copies/ml = 2) B. I-FABP by viral categories (<1000copies/ml=1, >1000 copies/ml = 2), C. sCD14 vs CD4 Count, D. I-FABP vs CD4 Count

In our cohort, I-FABP was modestly correlated with sCD14 (r = .223, p = 0.018) but did not show any association with CD4 count (r = .007, p = 0.939, Figure 6), viral load (r = .014, p = 0.885, Figure 6), or any of the T-cell subsets (data not shown). In addition, I-FABP levels did not correlate with HAZ (r = -.003), p = 0.968) or WAZ (r = .036), p = 0.707) values. On the other hand, sCD14 levels were associated with other variables, including an inverse correlation with CD4 count (r = -.21, p = 0.03, Figure 6), a positive correlation with the CD4 central memory subset (r = .20, p = 0.04), but not other T cell subsets (data not shown). sCD14 levels were identical in subjects stratified by low and high viral load (Figure 6), but statistically significantly higher in older aged (Figure 5) and in female (Figure 5) patients. Finally, sCD14 was not

associated with either HAZ (r = .07, p = 0.48) or WAZ (r = .08, p = 0.42). In summary, we observed detectable levels of I-FABP and sCD14, but the associations between these variables and those of CD4 count, viral load or T cell activation were either not apparent or only modest in comparison with many previous studies in adults.

Discussion

We evaluated a large cohort of HIV+ Ethiopian school age children for growth parameters, immune status, viremia, microbial translocation and immune activation. Our key findings were: 1) A large percentage (30%) of this cohort demonstrated growth failure and this was associated with changes in the distribution of T-cell subsets, in particular CD8 naïve and effector memory T cells; 2) Despite mean CD4 counts within the normal range for school age children, 30% of the children exhibited virologic failure with lower CD4 counts, and abnormal indices of T cell activation and differentiation; and 3) We documented detectable levels of I-FABP and s-CD14, surrogates for microbial translocation, but these were not associated with markers of T-cell activation.

It has been estimated that over 90% of HIV infected children worldwide will show some degree of growth failure which may not improve with antiretrovirals alone (Lodha & Kabra, 2015). The children in our cohort demonstrated a higher percentage of growth failure (40%) when compared with a nutritional status assessment conducted among a random sample of 459 school age children in Addis Ababa, Ethiopia in 2014 which reported 30.9% undernourished with 19.6% stunted and 15.9% underweight (Degarege, Degarege, & Animut, 2015). HIV children have been documented to have lower daily caloric intakes, higher resting energy expenditures and higher total energy expenditures than healthy children (Lodha & Kabra, 2015).

Whereas HAZ and HAZ were not associate with CD4 count, viral load, or frequencies of activated (CD38+HLA-DR+) or proliferating (Ki-67+) T cells, they were associated with some T cell subsets defined by differentiation markers, in particular naïve and effector memory CD8 T cells. These association were apparent even after correcting for the influence of all other variables in a multivariate analysis (data not shown). While few studies have addressed phenotypic markers of T cell differentiation according to nutritional status as we have done here, the importance of nutrition on T cell development and function is well known. While a long list of macro and micro nutrients are known to impact the immune system (Allen, Peerson, & Olney, 2009; Chandra, 2002), two hormones of potential relevance to our finding of the association between HAZ and enhanced frequencies of CD8 naïve T-cells include leptin and growth hormone. Leptin is produced by adipocytes after food intake and circulating levels correlate highly with total body fat content. It has profound effects on many cells of the immune system and is important in preparing cells towards nutrient and energy requiring events (Cohen, Danzaki, & MacIver, 2017; Gerriets & MacIver, 2014). Levels of leptin decrease significantly in malnourished individuals, and at least one study has reported levels in associating with naïve T cells (Chen et al., 2010). Growth hormone is a key hormone for body growth but also for immune development (Holländer, Krenger, & Blazar, 2010; Welniak, Sun, & Murphy, 2002). Malnutrition is associated with deficiencies in growth hormone's down-stream effector ILGF-1, likely contributing to stunting as well as thymus atrophy and hence low output of naïve T cells (Fazeli & Klibanski, 2014). Future quantitation of leptin and growth hormone levels in HIV infected children may elucidate the potential contribution of these hormone deficiencies to

abnormal naïve T cell number or function.

The CD8 effector memory subset has increasingly been shown to be persisting at high levels even after many years of suppressive ART (Gracía et al., 2005; Tobin & Aldrovandi, 2013). This subset may be associated with elevated pro-inflammatory systemic cytokines, which in turn are associated with risk for long term complications of HIV therapy including cardiovascular disease, metabolic syndrome, osteoporosis and dementia (Deeks, 2011; Duprez et al., 2012). The finding that children with higher HAZ scores had lower levels of CD8 effector memory, as well as increased naïve CD8 T cells, suggests the potential importance of appropriate nutritional guidelines and interventions to potentiate optimal distribution of CD8 T cell subsets in chronic HIV patients on ART.

Several pediatric cohorts have reported normalization of CD4 counts following the initiation of ART to levels near those of normal children (Gracía et al., 2005; Tobin & Aldrovandi, 2013). In our cohort, the mean and median CD4 counts were within normal limits, and despite the fact that we did not have pre-ART CD4 counts for comparison, this is consistent with the possibility that most of our pediatric cases similarly normalized their CD4 counts with ART. However, we obtained clear evidence that nearly a third of patients had detectable viral load, with over 12% having viral loads greater than 10,000 copies/ml. Moreover, high viral loads were clearly associated with lower CD4 counts as well as with abnormalities in the percentages of markers of T cell activation and proliferation, including elevated frequencies of CD8 T cells well as depressed frequencies of naïve and increased frequencies of memory subsets. While it has been repeatedly observed that these T cell abnormalities typical of HIV infection do not

completely normalize after ART (Gracía et al., 2005; Resino et al., 2001), the association with viral load and depressed CD4 count suggests that these subjects either failed to respond to initial therapy or initially responded but are now experiencing signs of disease progression. While it is known that HIV infected children commencing anti-retroviral therapy often require many more months than adults to attain complete viral suppression (Mofenson et al., 2009), perhaps as a consequence of high thymic output providing a continuous supply of suitable viral host cells, all of our cohort had been on therapy at least 6 months, and the vast majority several years, more than enough time for an adequate regimen to lead to virologic suppression. Thus, a trivial explanation that patients with detectable viral loads are experiencing declining viral load and hence improving rather than worsening, is unlikely. It is very probable that a significant fraction of those with detectable viral loads harbor resistance mutations.

A recent study from Ethiopia indicated that two thirds of pediatric subjects failing therapy in the southern city of Hawassa had drug resistance mutations in at least two classes of ART (nucleoside and non-nucleoside reverse transcriptase inhibitors) (Tadesse et al., 2018), a clear indication for a switch to alternative therapy, even though such therapy is currently limited in the country. The most likely cause of virologic failure is suboptimal adherence, and efforts to improve adherence along with the availability of viral load testing, mutational analysis when appropriate, and availability of either directed or empiric second line therapy are high priorities for continued effective therapy for pediatric HIV patients.

The levels of I-FABP and sCD14 we detected are in line with other studies documenting microbial translocation (Bi et al., 2016; Wallet et al., 2010) and while not

the focus of this study, our finding of higher sCD14 levels in females and older children was of interest. We noted literature support for both increased and decreased levels of sCD14 in females compared to males (Mathad et al., 2016; Raj et al., 2009) with little explanation of relationships and the increased levels with age are in line with others findings of lower levels in infants and young children (Pilakka-Kanthikeel et al., 2012; Wallet et al., 2010) An important finding in this pediatric HIV cohort was the lack of association between surrogates of translocation, sCD14 and I-FABP, and markers of disease progression or severity. Although the original research on the contribution of microbial translocation to immune activation in HIV pathogenesis observed strong correlations between markers of translocation, T cell activation, and indices of disease progression such as CD4 count and viral load, not all subsequent studies have observed clear associations (Brenchley et al., 2006). For example, a study in S. Africa reported a decrease in indices of T cell activation after ART among HIV adults but no change in levels of sCD14 thought to be primarily secreted upon direct interaction with monocytes lineage cells (Cassol et al., 2010). Similar findings were reported among a cohort of pediatric HIV patients in the US (Wallet et al., 2010). Our results of sCD14 as well as I-FABP, another surrogate molecule which is released upon enterocyte damage, are compatible with the latter two studies, although we are comparing patients on ART with either low or high viral load, the latter likely from treatment failure. Whereas we observed a modest inverse correlation between sCD14 and CD4 count, and with sCD14 and I-FABP, no other statistically significant associations were observed with either sCD14 and I-FABP and any of the other variables. We believe these studies underscore the complexity of mechanisms which contribute to microbial translocation and immune

activation. Different mechanisms may predominate in different settings and be influenced by different factors. Possible factors include diet, lifestyle behaviors, and underlying subclinical enteropathies.

The potential for diet can be appreciated with the demonstration that different diets have been shown to influence very significantly the distribution of either pro-inflammatory or anti-inflammatory commensal bacteria residing within the gut, the former more likely to exacerbate and the latter attenuate ongoing HIV enteropathy (Dillon et al., 2014; Dillon, Frank, & Wilson, 2016; Kau, Ahern, Griffin, Goodman, & Gordon, 2011). Certainly, diets among adult and children differ as do typical western diets from local traditional diets of sub-Saharan African countries such as Ethiopia.

Microbial composition has also been shown to be altered significantly by behaviors such as anal intercourse commonly practiced by men who have sex with men (Noquera-Julian et al., 2016). Other social behaviors common among HIV infected adults in western countries such as drinking, smoking, and illicit drug use may have systemic effects which potentially contribute to the extent of microbial translocation and immune activation and would unlikely be factors in the Ethiopian pediatric setting. Finally, a subclinical disease termed Environmental Enteropathy, with many similar pathologic features of HIV enteropathy, is a common condition in developing countries with compromised sanitation, a high prevalence of parasite infections and limited access to clean water (Crane, Jones, & Berkley, 2015). Although a limited number of studies in Ethiopia have documented risk factors for environmental enteropathy (Adane, Mengistie, Kloos, Medhin, & Mulat, 2017), how prevalent the disease is and how it may impact HIV enteropathy in pediatric patients with both conditions is unknown.

In summary, in this cohort of pediatric HIV in Addis Ababa, Ethiopia, 40% of the patients were observed to have suboptimal growth indices, and about 30% with incomplete viral suppression. While those with high viral loads exhibited reduced naïve subsets, typical T cell activation, proliferation and differentiation abnormalities, there was no association with viral load and growth indices. Rather, suboptimal growth indices were association with reduced naïve CD8 and enhanced effector memory CD8 T cells, after multivariate correction for other variables. Both of these findings, which have been reported in other pediatric HIV research, may contribute to the development of co-morbid disease. Finally, while we detected surrogate markers of microbial translocation, sCD14 and I-FABP, these markers showed minimal association with CD4 count, viral load or T-cell activation. These results underscore unique features of pediatric HIV disease and management in resource limited settings and suggest the continued need for further research.

REFERENCES

REFERENCES

Adane, M., Mengistie, B., Kloos, H., Medhin, G., & Mulat, W. (2017). Sanitation facilities, hygienic conditions, and prevalence of acute diarrhea among under-five children in slums of Addis Ababa, Ethiopia: Baseline survey of a longitudinal study. *PLoS ONE*, *12*(8). https://doi.org/10.1371/journal.pone.0182783

Allen, L. H., Peerson, J. M., & Olney, D. K. (2009). Provision of Multiple Rather Than Two or Fewer Micronutrients More Effectively Improves Growth and Other Outcomes in Micronutrient-Deficient Children and Adults. *Journal of Nutrition*, *139*(5), 1022–1030. https://doi.org/10.3945/jn.107.086199

Appay, V., & Sauce, D. (2008). Immune activation and inflammation in HIV-1 infection: causes and consequences. *The Journal of Pathology*, *214*(2), 231–241. https://doi.org/10.1002/path.2276

Bhatia, R., Ryscavage, P., & Taiwo, B. (2012). Accelerated aging and human immunodeficiency virus infection: emerging challenges of growing older in the era of successful antiretroviral therapy. *Journal of Neurovirology*, *18*(4), 247–255. https://doi.org/10.1007/s13365-011-0073-y

Bi, X., Ishizaki, A., Nguyen, L. V., Matsuda, K., Pham, H. V., Phan, C. T. T., … Ichimura, H. (2016). Impact of HIV Infection and Anti-Retroviral Therapy on the Immune Profile of and Microbial Translocation in HIV-Infected Children in Vietnam. *International Journal of Molecular Sciences*, *17*(8). https://doi.org/10.3390/ijms17081245

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., … Douek, D. C. (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature Medicine*, *12*(12), 1365–1371. https://doi.org/10.1038/nm1511

Cassol, E., Malfeld, S., Mahasha, P., van der Merwe, S., Cassol, S., Seebregts, C., … Rossouw, T. (2010). Persistent microbial translocation and immune activation in HIV-1-infected South Africans receiving combination antiretroviral therapy. *The Journal of Infectious Diseases*, *202*(5), 723–733. https://doi.org/10.1086/655229

Chandra, R. (2002). Nutrition and the immune system from birth to old age. - PubMed - NCBI. *Eur J Nutr*, *56*, 73–76.

Chen, J., Li, J., Lim, F. C., Wu, Q., Douek, D. C., Scott, D. K., … Louisiana Healthy Aging Study. (2010). Maintenance of naïve CD8 T cells in nonagenarians by leptin, IGFBP3 and T3. *Mechanisms of Ageing and Development*, *131*(1), 29–37. https://doi.org/10.1016/j.mad.2009.11.003

Cohen, S., Danzaki, K., & MacIver, N. J. (2017). Nutritional effects on T-cell immunometabolism. *European Journal of Immunology*, *47*(2), 225–235. https://doi.org/10.1002/eji.201646423

Crane, R. J., Jones, K. D. J., & Berkley, J. A. (2015). Environmental enteric dysfunction: An overview. *Food and Nutrition Bulletin*, *36*(1 0), S76–S87.

Deeks, S. G. (2011). HIV Infection, Inflammation, Immunosenescence, and Aging. *Annual Review of Medicine*, *62*(1), 141–155. https://doi.org/10.1146/annurev-med-042909-093756

Degarege, D., Degarege, A., & Animut, A. (2015). Undernutrition and associated risk factors among school age children in Addis Ababa, Ethiopia. *BMC Public Health*, *15*. https://doi.org/10.1186/s12889-015-1714-5

Dillon, S M, Lee, E. J., Kotter, C. V., Austin, G. L., Dong, Z., Hecht, D. K., … Wilson, C. C. (2014). An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal Immunology*. https://doi.org/10.1038/mi.2013.116

Dillon, S. M., Frank, D. N., & Wilson, C. C. (2016). The Gut Microbiome and HIV-1 Pathogenesis: A Two Way Street. *AIDS (London, England)*, *30*(18), 2737–2751. https://doi.org/10.1097/QAD.0000000000001289

Duprez, D. A., Neuhaus, J., Kuller, L. H., Tracy, R., Belloso, W., De Wit, S., … James D. Neaton1 for the INSIGHT SMART Study Group. (2012). Inflammation, Coagulation and Cardiovascular Disease in HIV-Infected Individuals. *PLoS ONE*, *7*(9), e44454. https://doi.org/10.1371/journal.pone.0044454

Fazeli, P. K., & Klibanski, A. (2014). Determinants of GH resistance in malnutrition. *The Journal of Endocrinology*, *220*(3), R57-65. https://doi.org/10.1530/JOE-13-0477

Franceschi, C., & Campisi, J. (2014). Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *69*(Suppl 1), S4–S9. https://doi.org/10.1093/gerona/glu057

Gerriets, V. A., & MacIver, N. J. (2014). Role of T cells in malnutrition and obesity. *Frontiers in Immunology*, *5*, 379. https://doi.org/10.3389/fimmu.2014.00379

Gracía, S. R., Noval, M. L., Rocha, R. C., González, M. G., Ruiz, V. C., & Muñoz-Fernández, M. (2005). Immune reconstitution in HIV-infected children on antiretroviral therapy. *Immunologia*, *24*(1), 44–54.

Harris, P. A., Taylor, R., Thielke, R., Payne, J., Gonzalez, N., & Conde, J. G. (2009). Research electronic data capture (REDCap)—A metadata-driven methodology

and workflow process for providing translational research informatics support. *Journal of Biomedical Informatics*, *42*(2), 377–381. https://doi.org/10.1016/j.jbi.2008.08.010

Holländer, G. A., Krenger, W., & Blazar, B. R. (2010). Emerging strategies to boost thymic function. *Current Opinion in Pharmacology*, *10*(4), 443–453. https://doi.org/10.1016/j.coph.2010.04.008

Hunt, P. W., Sinclair, E., Rodriguez, B., Shive, C., Clagett, B., Funderburg, N., … Lederman, M. M. (2014). Gut Epithelial Barrier Dysfunction and Innate Immune Activation Predict Mortality in Treated HIV Infection. *Journal of Infectious Diseases*, *210*(8), 1228–1238. https://doi.org/10.1093/infdis/jiu238

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., & Gordon, J. I. (2011). Human nutrition, the gut microbiome, and immune system: envisioning the future. *Nature*, *474*(7351), 327–336. https://doi.org/10.1038/nature10213

Kuller, L. H., Tracy, R., Belloso, W., Wit, S. D., Drummond, F., Lane, H. C., … for the INSIGHT SMART Study Group. (2008). Inflammatory and Coagulation Biomarkers and Mortality in Patients with HIV Infection. *PLoS Medicine*, *5*(10), e203. https://doi.org/10.1371/journal.pmed.0050203

Lodha, R., & Kabra, S. K. (2015). Health & nutritional status of HIV infected children. *The Indian Journal of Medical Research*, *141*(1), 10–12.

Mahnke, Y. D., Brodie, T. M., Sallusto, F., Roederer, M., & Lugli, E. (2013). The who's who of T-cell differentiation: Human memory T-cell subsets. *European Journal of Immunology*, *43*(11), 2797–2809. https://doi.org/10.1002/eji.201343751

Mathad, J. S., Gupte, N., Balagopal, A., Asmuth, D., Hakim, J., Santos, B., … Gupta, A. (2016). Sex-Related Differences in Inflammatory and Immune Activation Markers Before and After Combined Antiretroviral Therapy Initiation. *Journal of Acquired Immune Deficiency Syndromes 73*(2), 123–129. https://doi.org/10.1097/QAI.0000000000001095, 10.1097/QAI.0000000000001095

Mofenson, L. M., Brady, M. T., Danner, S. P., Dominguez, K. L., Hazra, R., Handelsman, E., … American Academy of Pediatrics. (2009). Guidelines for the Prevention and Treatment of Opportunistic Infections among HIV-exposed and HIV-infected children: recommendations from CDC, the National Institutes of Health, the HIV Medicine Association of the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the American Academy of Pediatrics. *MMWR. Recommendations and Reports: Morbidity and Mortality Weekly Report. Recommendations and Reports*, *58*(RR-11), 1–166.

Noquera-Julian, M., Rocafort, M., Guillen, Y., Rivera, J., Casadella, M., Nowak, P., …

Zeller, G. (2016). Gut Microbiota Linked to Sexual Preference and HIV Infection. *EBioMedicine*, *5*, 135–146.

Pilakka-Kanthikeel, S., Huang, S., Fenton, T., Borkowsky, W., Cunningham, C. K., & Pahwa, S. (2012). Increased Gut Microbial Translocation in HIV-infected Children Persists in Virologic Responders and Virologic Failures After Antiretroviral Therapy: *The Pediatric Infectious Disease Journal*, *31*(6), 583–591. https://doi.org/10.1097/INF.0b013e31824da0f5

Rainwater-Lovett, K., Nkamba, H., Mubiana-Mbewe, M., Bolton, M. C., Margolick, J., & Moss, W. (2014). Changes in Cellular Immune Activation and Memory T Cell Subsets in HIV-Infected Zambian Children Receiving HAART. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *67*(5), 455–462. https://doi.org/10.1097/QAI.0000000000000342

Raj, D. S., Carrero, J. J., Shah, V. O., Qureshi, A. R., Barany, P., Heimburger, O., … Stenvinkel, P. (2009). Soluble CD14 Levels, Interleukin-6, and Mortality Among Prevalent Hemodialysis Patients. *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation*, *54*(6), 1072–1080. https://doi.org/10.1053/j.ajkd.2009.06.022

Resino, S., Navarro, J., Bellón, J. M., Gurbindo, D., León, J. A., & Muñoz-Fernández, M. A. (2001). Naïve and memory CD4+ T cells and T cell activation markers in HIV-1 infected children on HAART. *Clinical and Experimental Immunology*, *125*(2), 266–273. https://doi.org/10.1046/j.1365-2249.2001.01612.x

Sandler, N. G., & Douek, D. C. (2012). Microbial translocation in HIV infection: causes, consequences and treatment opportunities. *Nature Reviews Microbiology*, *10*(9), 655–666. https://doi.org/10.1038/nrmicro2848

Smith, R. L., Boer, R. de, Brul, S., Budovskaya, Y., & Spek, H. van der. (2013). Premature and accelerated aging: HIV or HAART? *Frontiers in Genetics*, *3*. https://doi.org/10.3389/fgene.2012.00328

Somsouk, M., Estes, J. D., Deleage, C., Dunham, R. M., Albright, R., Inadomi, J. M., … Hunt, P. W. (2015). Gut epithelial barrier and systemic inflammation during chronic HIV infection: *AIDS*, *29*(1), 43–51. https://doi.org/10.1097/QAD.0000000000000511

Tadesse, B. T., Kinloch, N. N., Baraki, B., Lapointe, H. R., Cobarrubias, K. D., Brockman, M. A., … Brumme, Z. L. (2018). High Levels of Dual-Class Drug Resistance in HIV-Infected Children Failing First-Line Antiretroviral Therapy in Southern Ethiopia. *Viruses*, *10*(2). https://doi.org/10.3390/v10020060

Tobin, N. H., & Aldrovandi, G. M. (2013). Immunology of pediatric HIV infection. *Immunological Reviews*, *254*(1), 143–169. https://doi.org/10.1111/imr.12074

Wallet, M. A., Rodriguez, C. A., Yin, L., Saporta, S., Chinratanapisit, S., Hou, W., … Goodenow, M. M. (2010). Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T-cell activation following therapy: *AIDS*, *24*(9), 1281–1290. https://doi.org/10.1097/QAD.0b013e328339e228

Welniak, L. A., Sun, R., & Murphy, W. J. (2002). The role of growth hormone in T-cell development and reconstitution. *Journal of Leukocyte Biology*, *71*(3), 381–387.

WHO | Data and statistics. (n.d.). Retrieved December 7, 2016, from http://www.who.int/hiv/data/en/

CHAPTER 4

An evaluation of antihelminth treatment and an aloe enriched whey protein drink on measures of microbial translocation, immune activation and immune recovery in HIV+ children in Addis Ababa,

Abstract

Little research has evaluated immune activation in pediatric HIV cohorts, especially in developing countries. We evaluated the impact of a nutritional intervention on measures of microbial translocation, immune activation and immune recovery in a cohort of HIV+ children ages 4-11 in Addis Ababa, Ethiopia. We documented gains in height, weight and proliferating naïve CD4 T-cells as well as significant decreases in sCD14 within both the intervention and control groups. These gains may have been enhanced by antihelminth treatment and/or the benefit of perceived social support. Further research is warranted into the use of antihelminths in pediatric HIV and interventions aimed at increasing social support should be further explored. The aloe whey protein drink was safe and well tolerated.

Introduction

Immune activation and chronic inflammation has become a major focus of HIV disease research in both adults and children. Microbial translocation is a major contributing factor to immune activation, and occurs as a result of damage to the gut mucosa that begins early in the disease process but is reported to continue and have impacts on disease progression and immune reconstitution (Brenchley et al., 2006; Deeks, 2011; Klatt, Funderburg, & Brenchley, 2013). Little is known about levels of microbial translocation in children or how this relates to the development of chronic immune activation and inflammation in HIV+ children. The concern is that chronic low

grade inflammatory states may be initiated at young ages and while the magnitude of impact may not surface for years, early markers of co-morbid diseases such as cardiovascular disease, metabolic disorders and osteoporosis are starting to be documented in pediatric HIV populations (DiMeglio et al., 2013; Dimock et al., 2011, 2011; Lainka, Oezbek, Falck, Ndagijimana, & Niehues, 2002; Miller et al., 2012). Interventions aimed at attenuating immune activation both in adults and children are needed. Several trials with probiotics have been done or are underway in western countries, some showing promising results in reducing the indices of microbial translocation, immune activation and/or chronic inflammation (d'Ettorre et al., 2015; Falasca et al., 2015; Villar-García et al., 2015), and others with no demonstrable effect (Yang, Kelesidis, Cordova, & Khanlou, 2014). In resource limiting settings the long-term risks of chronic HIV infection are further confounded by malnutrition, particularly among pediatric HIV patients. Hence, there is a great need for interventions which both improve nutrition and reduce risk of chronic HIV complications such as immune activation profile. We chose an intervention incorporating both whey protein and Aloe Vera, both of which have shown positive benefits in HIV studies (Bounous, Baruchel, Falutz, & Gold, 1993; Olatunya, Olatunya, Anyabolu, Adejuyigbe, & Oyelami, 2012) and warrant further research involving HIV enteropathy. Whey protein is reported to be a highly bioavailable and well tolerated source of amino acids required for tissue healing and has been reported to have anti-inflammatory impacts in models of colitis (Krissansen, 2007; Smithers, 2008; Sprong, Schonewille, & van der Meer, 2010) . Aloe Vera has a long history of use as a topical wound healer and more recently has been evaluated in clinical trials using oral preparations (Langemead, L et al., 2004). We used a chocolate

flavored drink mix that incorporated both whey protein and aloe. We conducted a 12-week clinical trial with HIV+ children in Ethiopia exploring microbial translocation in a pediatric developing world context. Our goal was to evaluate a nutritional intervention in attenuating markers of microbial translocation and immune activation as well as their ability to impact measures of immune recovery.

Subjects and Methods

Subjects and study mechanics. Children between the ages of four and eleven, with documented HIV, who had been maintained on ART for a minimum of six months, were recruited between January and July 2017 from the pediatric out-patient clinic at Alert Hospital, Addis Ababa, Ethiopia. At enrollment, participants were screened for known co-morbidities including tuberculosis, hepatitis, liver disease, neurologic disease and cancers. The parents or guardians of all children provided written informed consent. All participants, families and clinic staff except the primary investigator remained blinded throughout the trial. The study proposal was approved by the Human Research Protection Program, Michigan State University (HRPP-MSU), as well as by the Alert/AHRI Ethics Review Committee (AAERC), the Ethiopian National Research Ethics Review Committee (ENRERC), and the Ethiopian Food, Medicine and Health Administration and Control Authority (EFMHACA).

Following enrollment blood samples were collected from all participants which was followed by the administration of a onetime dose (2-200mg tablets) of Albendazole (Eapozole, East African Pharmaceutical Pvt. Ltd. Co., Addis Ababa, Ethiopia) or 20ml Albendazole suspension (Alwo, Leben Laboratories, Pvt, Ltd, Mumbai, India). Additional doses were given to all family members. Participants were then supplied with a week's

supply of drink mix in individual servings along with directions for mixing which included an emphasis on the use of boiled or bottled water for reconstitution. A parent or guardian returned to the clinic weekly to obtain the next week's supply of drink mix and complete a brief health history on the child which also documented number of unused drink servings.

Interventions. Aloe Enriched Whey Protein Drink- Two hundred and fifty mg of Aloe Vera powder (BiAloe, Lorand Labs, Houton, TX, USA) was added to 2 tablespoons (TBLS) of chocolate-flavored whey protein mix (Combat Powder, MusclePharm, Denver, CO, USA). This serving size is approximately 1/3 of an adult serving and provides approximately 45 calories, 250 mg of aloe, and 8 gm of protein.

Chocolate Drink Mix- As an isocaloric comparison, Nesquik™ chocolate drink powder (Nestlé, Glendale, CA, USA) mixed with nonfat dry milk powder (Mix and Drink, Saco, USA), in a 2:1 ratio was packaged in 2 TBLS servings which provided approximately 45 calories and 0.8 gm protein.

Individual servings were placed into 3x3 zip lock bags and labeled with group number. A week's supply of these baggies was distributed along with a plastic cup with lid and instructions for mixing at enrollment and at each weekly follow up visit any unused baggies from the week before were collected and a new week's supply given. Parents and guardians were instructed to mix drink powders with 4-8 oz of bottled or boiled water. Plastic cups with lids were provided to allow for thorough mixing and parents were instructed to wash these between uses. New cups were provided each month.

Albendazole- Two 200-mg chewable Albendazole tablets (Eapozole, East African

Pharmaceutical Pvt. Ltd. Co., Addis Ababa, Ethiopia) or 20ml Albendazole suspension (Alwo, Leben Laboratories, Pvt, Ltd, Mumbai, India) were provided to all study participants, as well as to family members living in the same household, as a one-time dose to treat intestinal parasites. Dosing is the same for adults and children. This was used to prevent confounding of intestinal parasites on measures of immune stimulation as intestinal parasites are well documented for contributing to immune stimulation (Borkow et al., 2000; Kalinkovich et al., 1999; Walson, Herrin, & John-Stewart, 2009).

* All children were maintained on their established treatment regimens throughout the trial.

Anthropometric status. At enrollment, all children were assessed for height, weight and mid-upper arm circumference (MUAC) by trained nurses. Heights were measured on a platform stadiometer with the child's shoes removed, their back to the height rod and flat headpiece loosely resting on the flat of their head and measurements recorded to the nearest 0.2 centimeter. Weights were measured on a digital scale with shoes and outer clothing removed and measured to the nearest 0.1 kilograms. Heights and weights were converted into height for age (HAZ), weight for age (WAZ), using the Quesgen online pediatric z-score calculator (*www.quesgen.com/BMIPedsCalc.php*) according to WHO-child growth parameters. A z-score of 0 represents the median based on the WHO age/sex reference data. A z-score of -1 represents a measurement that is 1 standard deviation below the reference population. To determine if there is a slowing of growth as HIV children age we further stratified our cohort into 2 groups by age such that ages 8 and under = 0 and ages over 8 = 1 and did further growth evaluation. Study data were collected and managed using REDCap electronic data

capture tools hosted at Michigan State University. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies (Harris et al., 2009).

Clinical laboratory results. At enrollment, 8-10 ml of blood was drawn from each participant and collected in BD EDTA and clot Vacutainer tubes. Viral loads as well as CD4 counts and complete blood counts (CBC) were performed by hospital laboratory technicians at Alert Hospital. Viral loads were assayed using an Abbot n2000sp for extraction and Abbot m2000rt for quantification of viral copies/ml. CD4 counts were done using a BD FACSounter, while CBCs were run using a Sysmek Kx-21. Remaining plasma and serum was aliquoted into 1.5ml Eppendorf tubes and stored at -80C for ELISAs and further assays.

Lymphocyte subsets. Immune reconstitution was evaluated based on CD4 counts as well by quantification of recent thymic emigrants expressing CD31 and proliferating naïve CD4 subsets using Ki-67, an intracellular marker of homeostatic proliferation. Cellular immune activation was quantified based on T-cell phenotype and expression of surface markers HLA-DR and CD38 on CD8 T-cells. Separated blood cells from EDTA centrifuged tubes were reconstituted in appropriate amounts of phosphate buffered saline (PBS) and stained for surface and intracellular markers using fluorochrome conjugated antibodies against CD4-PE, CD8-BV421, CD31-BV510, CD38-PECy7, CD45RA-APC-H7, CD62L-APC, HLA-DR-PerCP5.5 and Ki-67-FITC (BD Biosciences) according to the protocol outlined below. Three hundred microliters of cells mixed in PBS was aliquoted into polystyrene flow cytometry tubes followed by a cocktail of optimized amounts of antibody stains (2.5-20µl) and incubated at room temperature for 20 minutes. This was followed by the addition of 2ml of FACS lysis buffer (BD

Biosciences, Catalog No.349202) for an additional 10 minutes at room temperature. The sample was then washed with 2ml FACS buffer (PBA + 1mg/ml serum bovine albumin and 1 mM EDTA) and centrifuged at 1700 RPMs x 5 minutes in a Beckman Alegra 5 Centrifuge. Following the wash, the supernatant was decanted, cells were resuspended and incubated with 500 µl of permeabilizing FACS solution (Perm-2, BD Biosciences, Catalog No.40973) at room temperature for 20 minutes. This was followed by another wash cycle with 3.5 ml FACS buffer followed by centrifuging at 2100 RPM for 12 minutes. After decanting and resuspension 2.5µl of anti-Ki-67 was added and incubated for an additional 20 minutes at room temperature followed by another wash with 4 ml FACs buffer and centrifuged at 2100 RPM x 12 minutes. Cells were then resuspended and fixed with 300 µl of 2% formaldehyde x 30 minutes followed by a final wash with FACS buffer. The stained and fixed cells were then stored at 4C in the dark until acquired into a FACS Canto 8 Color Flow Cytometer (BD FACSCantoTM II) according to manufacturer instructions using compensation microbeads from BD and Diva software. A minimum of 100,000 events per sample were collected for analysis. Lymphocytes were identified based on forward and side scatter (FSC-SSC). Frequencies and percentages of T-cell subsets were calculated from total counts of “parent” populations and were identified and quantified by gating strategies using FlowJo_v9.9.6 software.

Intestinal barrier function. Microbial translocation was evaluated using two surrogate markers namely, intestinal fatty acid binding protein (I-FABP), an intracellular protein released from damaged enterocytes that line the intestinal mucosal, and soluble CD14 (sCD14), a surface acute phase protein released by monocytes upon activation

by lipopolysaccharide (LPS) and other antigenic stimuli. The measurement of sCD14 has become an established indicator of microbial translocation and innate immune activation in HIV research involving both adults and children. Both I-FABP and sCD14 were quantified using ELISA kits according to manufacturer directions (Hycult-Biotech, Netherlands). Briefly, all samples and reagents were brought to room temperature and plasma aliquots were diluted 2X for I-FABP assays and 320X for sCD14 assays.

Statistical analysis. All data was analyzed using JMP software v6 (SAS, Cary, NC). Descriptive statistics were used to report demographic and clinical data. Nominal data were reported by percentages and frequencies whereas continuous data described by means, standard deviation, median and interquartile ranges. Comparison of outcome variables was determined using either paired t-tests or ANOVAs for variables with normal distributions and Wilcoxon signed rank test for variables with non-normal distributions. Correlation analyses were done using Pearson's Correlations for parametric and Spearman's Correlations for non-parametric data. 2-way ANOVA was used to test for statistically significant differences in outcome variables both across treatment time (before vs after therapy) as one categorical variable and across treatment cohort (Aloe/Whey vs Control) as the other categorical variable. Significant differences between groups was determined at the 0.05 level of significance. Each outcome variable was independently assessed. Because we considered this exploratory research, no corrections were made for multiple comparisons.

Results

Baseline characteristics. One hundred twenty children between the ages of 4-11 years old were enrolled between January 2–May 2, 2017 and randomized to control

or intervention group (mean 8.1, 8.0 respectively). One hundred eleven children completed the 12-week trial. Four children withdrew during the first week, four additional were lost to follow up despite repeated phone calls, and one was dropped by investigators due to severe malnutrition that warranted a more calorie dense-nutritional supplement and monitoring. Groups were similar in makeup as demonstrated in Table 1. Compliance throughout the trial was monitored weekly by recording numbers of unused drink packets and collecting returned packets. Out of a total of 9324 daily packets issued to those who completed the trial, 107 were reported as unused for various reasons.

Variable	Control (Nesquick)	Intervention (Aloe+Whey)
Number recruited	57	63
Number completed	51	60
Sex (male, female)		
male	26	38
female	25	22
Age	8.1 (1.8)	8.1 (2.0)
Weight for Age (WAZ)	-1.6 (1.0)	-1.6 (1.0)
Height for Age (HAZ)	-1.6 (1.0)	-1.54 (1.1)
Baseline BMI	14.9(1.1)	15.1(1.6)
CD4 cnt(cells/mm3)	1012 (432)	890 (438)
Viral Load (copies/ml)	47	54
Median [range]	0 [0-140175]	0 [0-170355]
Mean (Std Dev)	6294 (23599)	13766 (37629)
No with > 1000	*8 (47)	*16 (54)
No with > 10000	*4 (47)	*9 (54)
HGB(gm/dl)	13.5 (0.9)	13.9 (1.7)
SGOT(units/liter)	31.5 (6.4)	33.1 (9.7)
SGPT(units/liter)	25.2 (8.9)	25.6 (10.4)
Creatinine(mg/dl)	0.4 (0.1)	0.4 (0.1)

Table 2. Participant descriptions at baseline. *Based on number with reported viral load at baseline.

All of the children enrolled in this project had been maintained on a regimen that

incorporated at least 3 drugs for at least 6 months. Eight different treatment regimens were reported with the regimen of AZT/3TC/NVP being the most prevalent at (46.7%) followed by AZT/3TC/EFV at 32.5% (Table 2.). Twenty-two children were also receiving Cotrimoxazole daily as prophylaxis to prevent secondary infections. The mean length of time on antiretroviral therapy among all participants at enrollment was greater than 5 years (mean 5.2$\pm$2.5, median 5.6 years). No significant difference in CD4 counts or viral loads was noted based on treatment regimen (data not shown).

		Frequency	Percent
Valid	ABC/3TC/EFV	4	3.3
	ABC/3TC/KALETRA	3	2.5
	ABC/3TC/NVP	2	1.7
	AZT/3TC/ATV(r)	2	1.7
	AZT/3TC/EFV	39	32.5
	AZT/3TC/KALETRA	12	10.0
	AZT/3TC/NVP	56	46.7
	d4T/3TC/NVP	2	1.7
	Total	120	100.0

Table 3. ART Regimens (ABC – abacavir, 3TC- lamivudine, EFV- efavirenz, NVP - Nevirapine, AZT- zidovudine, ATV – atazanavir, ATV(r)- Atazanavir boosted with ritanovir, d4T – stavudine)

Undernutrition was evident across groups at baseline with mean height for age z-scores (HAZ) and weight for age z-scores (WAZ) in both groups less than -1.5. Forty percent of our cohort was determined to be stunted or undernourished as defined by HAZ or WAZ <-2 with a total of 31.7% who were underweight and 32.5% who were stunted. CD4 counts among both groups spanned a broad range with mean of the control group at 1012(432) and in the Aloe/whey group 890(438). While the large

majority of children demonstrated viral loads below detection, 23.8% of children demonstrated viral loads above 1,000 copies/ml at enrollment with viral loads in thirteen children being over 10,000 copies/ml.

Adverse event and safety tracking. Five children throughout the trial presented with symptoms consistent with potential adverse events and were evaluated by clinic practitioners: One reporting generalized itching with a history of the same that responded to Loratadine, one presented with a two day history of vomiting/diarrhea with headache and chills which resolved without treatment, three reporting abdominal symptoms of cramping and diarrhea with one resolving spontaneously and two being treated (Cotrimoxazole or Metronidazole) based on stool sampling. The symptoms of all five children were determined to not be related to study interventions and no child was removed from the trial. Adverse event reporting forms were completed and reported to the chair of the data safety and monitoring board (DSMB).

We quantified and compared liver enzymes (AST, ALT) and kidney function (creatinine) as well as hemoglobin measures pre and post supplementation to monitor for safety of study interventions. Levels were within the normal reference ranges at baseline and at follow-up (data not shown).

CD4 counts and viral loads. Until recently the mainstay of HIV treatment monitoring in Ethiopia has been CD4 counts which provide guidelines for when to initiate therapy as well as a measure of immune reconstitution. CD4/CD8 ratios are also used in some settings as a more accurate means of measuring immune reconstitution. Mean CD4 counts in both groups were within normal pediatric ranges and we saw no significant change in either CD4 counts or viral loads between groups or time periods

nor were any changes observed in CD4/CD8 ratios between groups (Figure 7).

Intestinal barrier integrity. The main aim of this trial was to evaluate whether dietary supplementation with an aloe enriched whey protein drink would support tissue healing of the gut mucosa as evidenced by lowered levels of surrogate markers of microbial translocation (I-FABP and sCD14). We hypothesized that intervention with Aloe/Whey nutritional supplements would result in a significant reduction in levels of both I-FABP and sCD14.

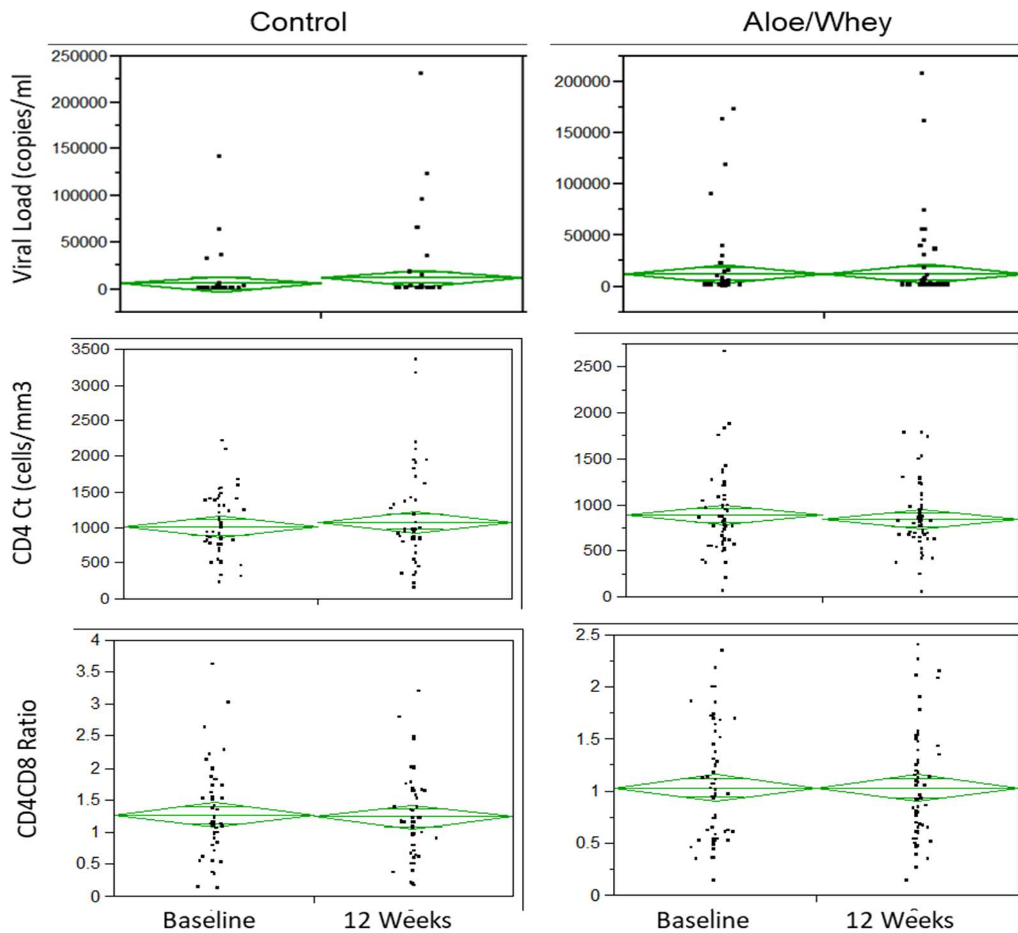

Figure 7. Comparison of Viral Load, CD4 Counts and CD4CD8 Ratios.

As depicted in Figure 7, there was no decrease, but a slight increase in the mean levels of Intestinal Fatty Acid Binding Protein (I-FABP) between time points in both

groups which was more notable in the control group (544 pg/ml vs 599 pg/ml) over the intervention group (573 pg/ml vs 595 pg/ml). These changes in the levels of I-FABP however, did not differ significantly between the two treatment groups (p = 0.84).

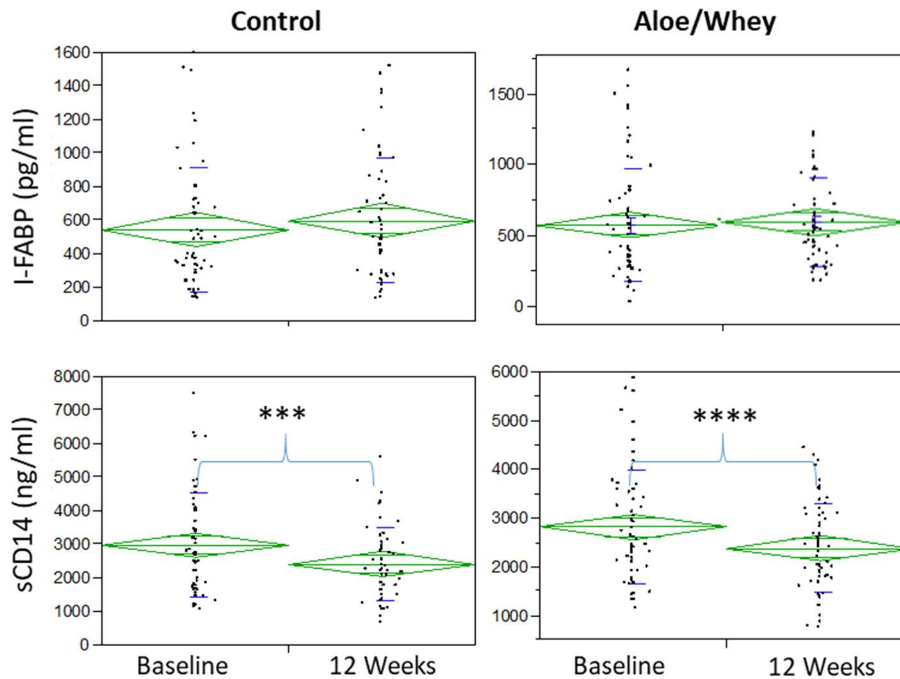

Figure 8: Comparison of I-FABP and sCD14. Baseline and 12 weeks by groups. (*** = p <0.001, **** = p ≤0.0001)

Plasma levels of soluble CD14 (sCD14), as shown in Fig. 8, did significantly decrease after the 12-week therapy, for both Aloe/Whey (p = 0.0001) and control (p = 0.0009) groups. The decrease in sCD14 did not differ between the two treatment groups, however (p = 0.854), indicating the Aloe/Whey was not superior to control.

Anthropometric measurements. We hypothesized that the Aloe/Whey treatment group would respond with some measure of growth increase over control. Both groups showed gains in weight and height over the 12-week period (Figure 9). Whereas the Aloe/Whey group demonstrated statistically significant weight gain with treatment (p = 0.03), the control group achieved significant height gains after

intervention (p = 0.007). Tests of significance between the groups, however indicated that neither was superior to the other for either WAZ (p = 0.743) or HAZ (p = 0.947). No significant change was found in height for weight (HWZ), BMI or mid upper arm circumference between groups (data not shown).

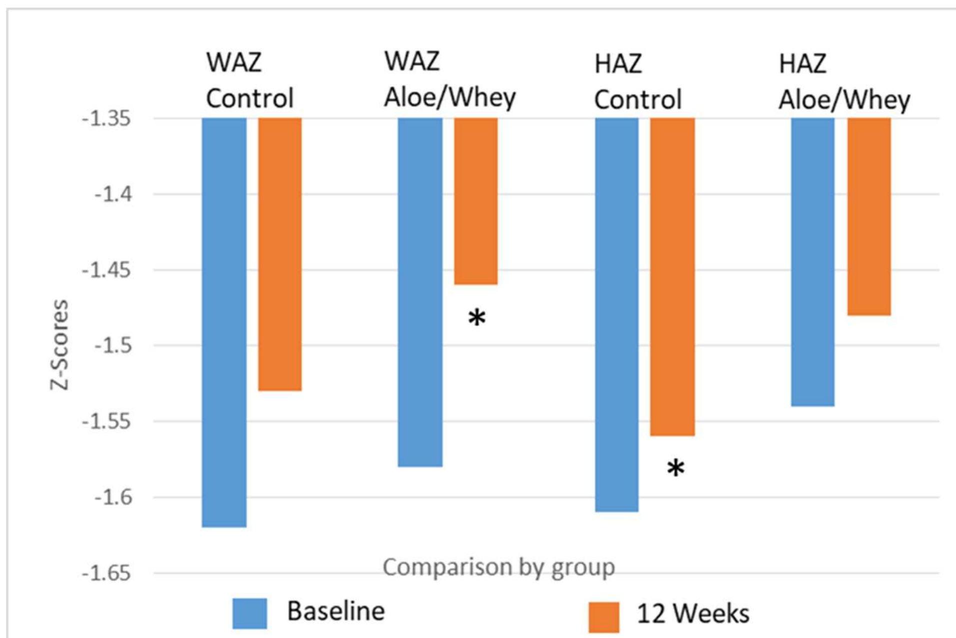

Figure 9. WAZ and HAZ change within groups. Control Values: Baseline vs 12-week WAZ -1.62(0.98) vs -1.52(0.92). Baseline vs 12-week HAZ -1.61(0.99) vs -1.56(0.94). Aloe/Whey Values: Baseline vs 12-week WAZ -1.57(0.99) vs -1.46(0.99). Baseline vs 12-week HAZ -1.54(1.06) vs -1.48(0.98). The gain in WAZ within the Aloe/Whey cohort was significant (p = 0.03), and the increase within HAZ among the control cohort was significant at (p = 0.007).

Proliferating naïve CD4 subsets. Following a rapid redistribution phase of CD4 reconstitution immediately after initiation of ART, improvements in CD4 levels increase only gradually. Hence, the nutritional potential on CD4 count improvement within our cohort, who were all on ART for at least 6 months, may be difficult to assess within the relatively short 12-week intervention period. We therefore evaluated subsets of CD4 T cells which might be more sensitive predictors of CD4 reconstitution in a longer term nutritional intervention. We thus quantitated by flow cytometry total naïve CD4 T cells,

recent thymic emigrant naïve CD4 T cells, as well as homeostatically regenerating naïve T-cell subsets. Figure 10 (panels A-F) provides a picture of the gating strategy used.

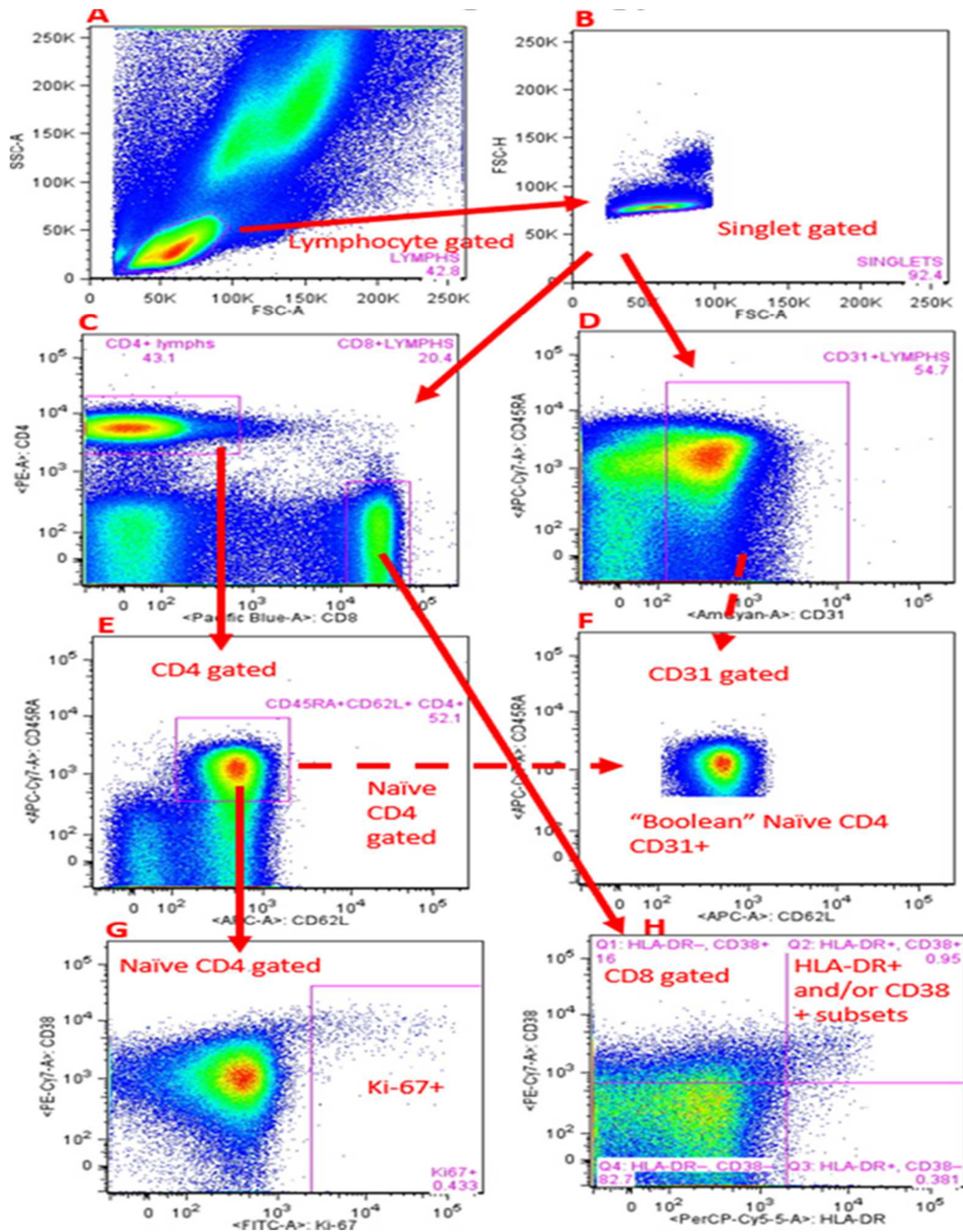

Figure 10 Gating strategy to define lymphocyte subsets. **A.** Lymphocyte gating, **B.** Singlet gate,**C.** CD4 and CD8 gates, **D.** CD31+ lymphocytes, **E.** Native CD4 gate, **F.** CD31+Naïve CD4 T cells, **G.** Ki-67+Naïve CD4 T cells, **H.** CD8 T-cells expressing CD38 and/or HLA-DR molecules

The naïve subsets were defined as CD45RA+CD62L+CD4 +, the regenerating naïve T-cells were naïve CD4 T cells expressing the proliferation marker Ki-67, recent thymic emigrants (RTE) were defined as CD31+ naïve CD4 T cells. As shown in Figure 11, while there was no appreciable difference in the frequencies of naïve CD4 T cells in either Aloe/Whey or control groups, we did observe significant increases in the levels of naïve regenerating (Ki67+CD62L+CD45RA) CD4+ T-cells in both the control arm (p = 0.003) and the Aloe/Whey group (p = 0.007).

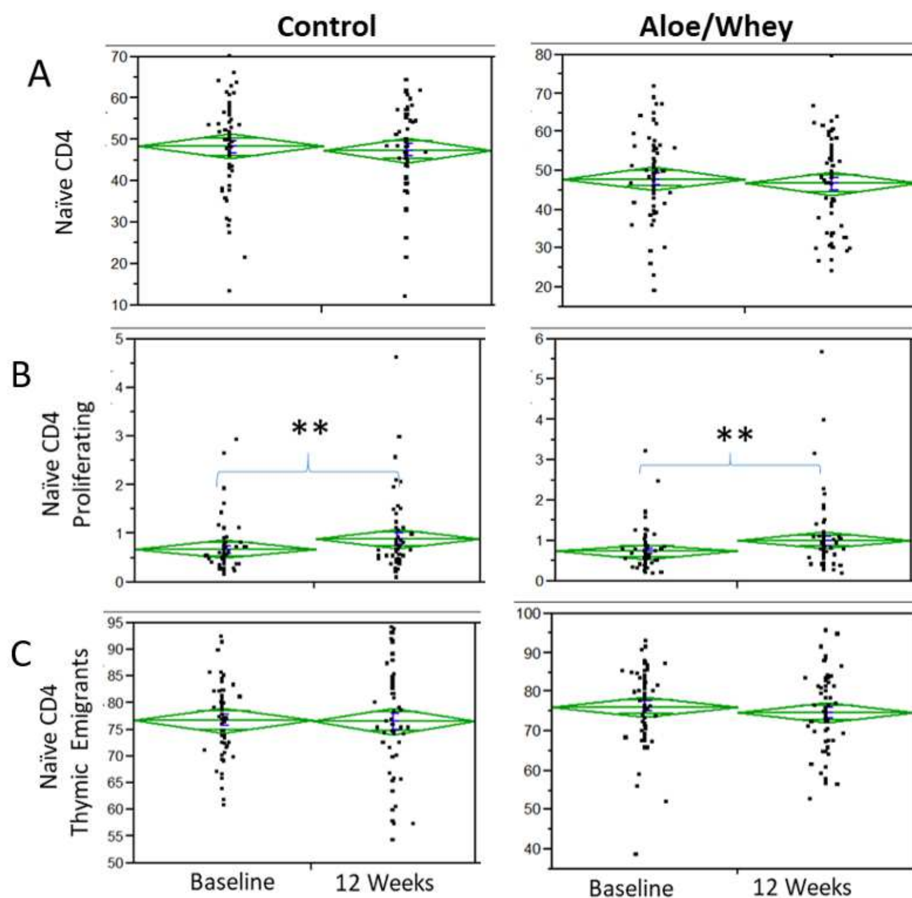

Figure 11. Comparison of subsets of CD4 T- cells with trial groups. A) CD45RA+CD62L+ (Naïve CD4+ T cells), p = 0.07 (Control), 0.14 (Aloe/Whey); B) CD45RA+CD62L+Ki67+ (Naïve Regenerating CD4+ T cells) p = 0.003 (Control), 0.007 (Aloe/Whey); C) CD45RA+CD62L+CD31+ (recent thymic emigrants CD4+ T cell) p = 0.91 (Control), 0.31 (Aloe/Whey. Tests across control and aloe/whey groups for populations A, B, C were p = 0.72, , 0.52, and 0.78, respectively.)

The improvement seen in the Aloe/Whey cohort was not superior to that of the control group (p = 0.52) (Fig 11). No differences were found in the recent thymic emigrants (CD4+CD45RA+CD62L+CD31+) subset after treatment in either group.

Activated CD8 T-cells. T-cell activation has been reported to be associated with microbial translocation in adult HIV studies, so we were interested to see if the nutritional intervention had an impact in our cohort. Comparison of CD8 subsets expressing activation markers CD38 and HLA-DR before and after therapy among the two groups was performed by flow cytometry (see Figure 10 for gating strategy). We hypothesized that activated CD8 T cells would be reduced after Aloe/Whey therapy.

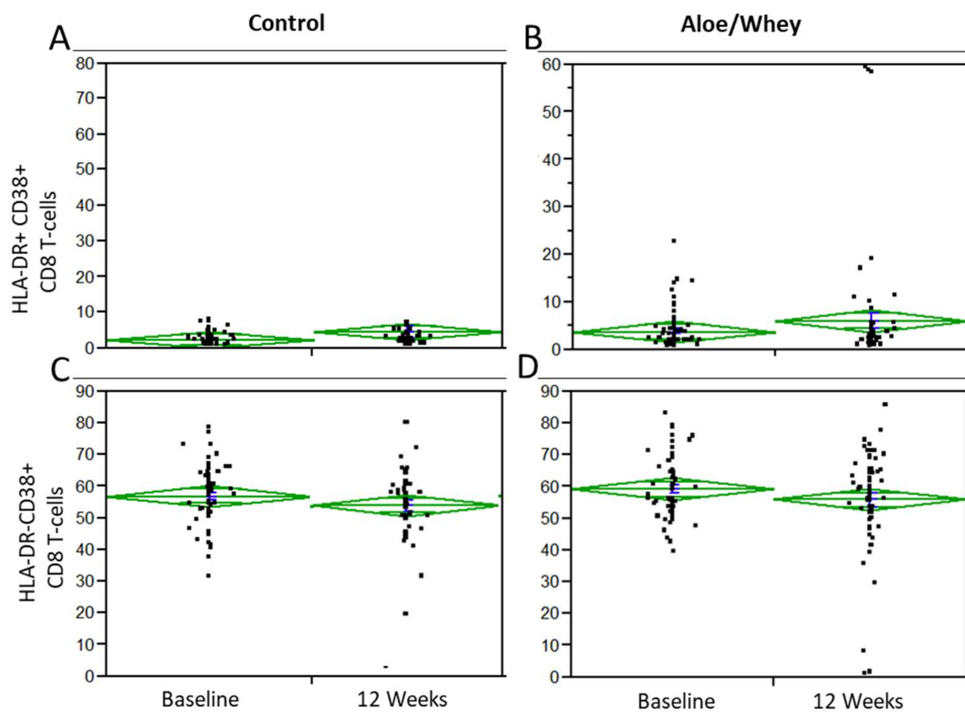

Figure12. Comparison of CD8 activation subsets by groups. A) HLA-DR+CD38+CD8 T-cells Control p=0.41; B) HLA-DR+CD38+CD8 T-cells Aloe/Whey p=0.17 C) HLA-DR-CD38+CD8 T-cells Control p=.80 D**)** HLA-DR-CD38+ T-cells Aloe/Whey p=0.29. Tests across control and aloe/whey groups were p = 0.17 (HLA-DR+CD38+ CD8 T cells), and p = 0.40 (HLA-DR-CD38+ CD8 T cells).

As depicted in Figure 12, a slight increase in CD38+DR+ and a slight decrease in

CD38+DR- CD8 T cell subsets was observed in both Aloe/Whey and Control groups, but none of these differences were statistically significant.

Discussion

Microbial translocation and associated chronic immune activation has only been explored in a few cohorts of HIV+ children and to our knowledge, no trials have evaluated a nutritional intervention to attenuate microbial translocation and chronic immune activation and promote CD4 reconstitution in children. This 12-week clinical trial allowed for the collection and analysis of data from a group of HIV+ children in a developing world context who have been stably maintained on treatment. We had high rates of retention and compliance throughout the trial which may have been boosted by the sweet, chocolate flavored interventions.

We hypothesized that relative to isocaloric control, the Aloe/Whey nutritional mix would increase measures of height and weight, decrease surrogates of microbial translocation sCD14 and I-FABP, and increase measures of de novo naïve CD4 generation, naïve Ki67+ and CD31+ recent thymic emigrants. We observed: 1) There were expected changes in several of the outcome variables, but when changes occurred they occurred both in the Aloe/Whey arm as well as the placebo control arm; 2) Of the markers of microbial translocation, while sCD14 decreased, the other, I-FABP was unchanged, and we did not see any associated changes in activation markers on T cells ; 3) Of the measures of de novo naïve CD4 T cell generation, Ki67+ naïve CD4 cells increased, whereas thymic emigrants did not.

The simultaneous change in both Aloe/Whey and isocaloric control could have been due to at least three possibilities. First, the study was designed to specifically

address a nutritional benefit of Aloe/Whey, and we pretreated all individuals in the study with anti-helminths in order to minimize the potential confounding effect of these microbes. The composite literature on the impact of helminth infections on nutritional status, cognition as well as the immune system has included studies on both HIV negative and positive individuals and is highly contradictory. Much of the early literature supported mass anti-helminth campaigns, but more recent studies and analyses have challenged these assertions, particularly with regard to growth, minimization of anemia and cognitive benefit (Taylor-Robinson, Maayan, Soares-Weiser, Donegan, & Garner, 2015). There is considerable evidence that helminth infections induce immune responses, including T cell activation (Borkow et al., 2000). Many studies have reported modest benefits among HIV patients of deworming on CD4 counts and viral load (Means, Burns, Sinclair, & Walson, 2016), though inconstancies have been reported which may relate to the distribution of worm species among study populations (Sangare, Herrin, John-Stewart & Walson, J, 2011). Others have shown higher frequencies of activated T cells in association with specific helminth infections (Borkow et al., 2000), but few of these have reported activation marker reversal with anti-helminth treatment (Chachage et al., 2014). Regarding studies of microbial translocation, one study showed an increase in plasma sCD14 associated with hookworm infections, reversed with therapy (Parakkal et al., 2012). Two reports studied the impact of strongyloides infection on plasma sCD14 and arrived at opposite conclusions (George et al., 2014; Rajamanickam et al., 2017). An additional paper reporting on hookworm, as well as ascaris and trichuria, two species more commonly identified in Addis Ababa (de Carneri, Di Matteo, & Tedla, 1992), observed that T cell activation was seen with ascaris and

trichuria but not hookworm, and activation markers did not appreciably change after therapy (Chachage et al., 2014). It is also noteworthy the much of the key cited literature on the convincing benefits of deworming is based on screening and treatment of proven infected individuals, rather than a mass deworming campaign without prescreening, despite advocating for the latter (Taylor-Robinson et al., 2015; WHO, 2017). In the context of our study, while literature exists which might easily explain our findings, given the multitude of contradictions, there appears to be no compelling evidence to support antihelminths as a sole contributor.

A second plausible explanation for the results we obtained may have been perceived social support. HIV is still very stigmatized in Ethiopia as elsewhere and infected individuals are marginalized. The weekly follow up visits that our trial design utilized allowed for frequent interactions. This alone may have had a very positive impact on both the children and the mothers which could in turn have affected appetite and food intake (McIntosh, Shifflett, & Picou, 1989). Weekly comments were documented from patients and although not systematically quantified, it was clear that numerous parents of children from both groups made comments about their child's appetite improving, about them eating better and that they felt their child was gaining weight. These anecdotal observations are consistent with the very encouraging weight and height gains which we did document over a relatively short duration of supplementation; such gains are unlikely to be attributable to the small increase to caloric intake (approximately 45 calories) each day. Social support, including communication and positive interactions with health care providers, has been shown to improve drug adherence as well as treatment response (Cummings et al., 2014; Kundu, Samanta, Sarkar, Bhattacharyya, & Chatterjee, 2012; van Servellen & Lombardi, 2005). Thus, we believe more research studies

in this context should be undertaken to implement and evaluate social support systems.

A third possibility is that there are effects of Aloe/Whey that are superior to that of the control group which were either not revealed during the study period or have not as yet been studied. The differences we did observe were small, and quite probably, a much longer period may have more clearly revealed differences in some variables like weight or height gain, as well as CD4 recovery. Moreover, anti-inflammatory and anti-oxidant activities have been associated with both Aloe Vera and Whey Protein (Budai, Varga, Milesz, Tozser, & Benkő, 2013; Jiang et al., 2016; Kent, Harper, & Bomser, 2003; Sprong et al., 2010), and these and other outcome variables may be considered in future studies.

Several studies have reported sustained levels of I-FABP and/or sCD14 (Brenchley et al., 2006; Hunt et al., 2014; Prendergast et al., 2017) in HIV cohorts following initiation of ART, so it was significant to document declines of sCD14 in both the Control and Aloe/Whey groups. The lack of parallel decline in levels of I-FABP is not clear, but the plasma levels of the two markers may ultimately result from separate processes. While I-FABP is strongly associated with epithelial cell death, which when excessive can lead to influx of lumen bacteria or their products (Bischoff et al., 2014). Conversely, sCD14 is secreted in response to many stimuli, including translocated bacterial products such as endotoxin, which in theory could result from altered epithelial tight junctions, and not necessarily secondary to breaches due to epithelial death (Bischoff et al., 2014). Other researchers have reported a lack of association between these markers (Kelesidis, Kendall, Yang, Hodis, & Currier, 2012).

Many studies evaluating microbial translocation report associated CD8 T cell

activation. Nonetheless, in our study, while supplementation decreased sCD14, we did not observe an accompanying decline in activated CD8 T cells. Here, too, literature can be found which does not support the strict association of these two biomarkers (Cassol et al., 2010; Wallet et al., 2010). While the appearance of sCD14 is strongly related to monocyte activation in response to bacterial products such as endotoxin, the mechanism of associated T cell activation is not clear, but is apparently not related to bacteria-reactive T cells interacting with monocyte/macrophage lineage cells (Wallet et al., 2010; Zimmermann et al., 2015).

Two mechanisms have been elucidated which result in de novo naïve T cells, recent emigration from the thymus, and homeostatic proliferation of naïve T cells (Boyman, Létourneau, Krieg, & Sprent, 2009; Kohler & Thiel, 2009). Thymic differentiation is the major mechanism early in life for generating newly matured naïve T cells. Homeostatic mechanisms occur peripherally outside of the thymus, is independent of foreign antigen, requires cell contact with MHC bearing professional phagocytes, such as dendritic cells, and requires other cytokines including IL-7 and/or IL-15 (Boyman et al., 2009). Our findings of increased naïve proliferating, but no change in recent thymic emigrant subsets, supports that these are two largely independent processes. Incomplete or slow restoration of CD4 T cells has been a problem in both pediatric and adult HIV, and has prompted research with experimental modalities, such as IL7 administration to boost numbers of naïve T cells in HIV patient on ART (Levy et al., 2012). Assays to detect thymic emigrants and naïve CD4+ Ki67 positive proliferating cells, provide potentially more sensitive means to explore interventions to reconstitution CD4 T cells.

In summary, we obtained encouraging results of an interventional nutritional trial including weight and height gains, a decrease in the levels of surrogates of microbial translocation, and an increase in regenerating CD4 T cells. The impacts of the nutritional intervention themselves, as well as anti-helminth treatment, and social support are discussed, and suggested as the topics for continued research in this area.

REFERENCES

REFERENCES

Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.-D., Serino, M., … Wells, J. M. (2014). Intestinal permeability--a new target for disease prevention and therapy. *BMC Gastroenterology*, *14*, 189. https://doi.org/10.1186/s12876-014-0189-7

Borkow, G., Leng, Q., Weisman, Z., Stein, M., Galai, N., Kalinkovich, A., & Bentwich, Z. (2000). Chronic immune activation associated with intestinal helminth infections results in impaired signal transduction and anergy. *Journal of Clinical Investigation*, *106*(8), 1053.

Bounous, G., Baruchel, S., Falutz, J., & Gold, P. (1993). Whey proteins as a food supplement in HIV-seropositive individuals. *Clinical and Investigative Medicine. Médecine Clinique Et Experimentale*, *16*(3), 204–209.

Boyman, O., Létourneau, S., Krieg, C., & Sprent, J. (2009). Homeostatic proliferation and survival of naïve and memory T cells. *European Journal of Immunology*, *39*(8), 2088–2094. https://doi.org/10.1002/eji.200939444

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., … Douek, D. C. (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature Medicine*, *12*(12), 1365–1371. https://doi.org/10.1038/nm1511

Budai, M., Varga, A., Milesz, S., Tozser, J., & Benkő, S. (2013). Aloe vera downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages. *Molecular Immunology*, *56*, 471–479.

Cassol, E., Malfeld, S., Mahasha, P., van der Merwe, S., Cassol, S., Seebregts, C., … Rossouw, T. (2010). Persistent microbial translocation and immune activation in HIV-1-infected South Africans receiving combination antiretroviral therapy. *The Journal of Infectious Diseases*, *202*(5), 723–733. https://doi.org/10.1086/655229

Chachage, M., Podola, L., Clowes, P., Nsojo, A., Bauer, A., Mgaya, O., … Geldmacher, C. (2014). Helminth-Associated Systemic Immune Activation and HIV Co-receptor Expression: Response to Albendazole/Praziquantel Treatment. *PLOS Neglected Tropical Diseases*, *8*(3), e2755. https://doi.org/10.1371/journal.pntd.0002755

Cummings, B., Gutin, S. A., Jaiantilal, P., Correia, D., Malimane, I., & Rose, C. D. (2014). The Role of Social Support Among People Living with HIV in Rural Mozambique. *AIDS Patient Care and STDs*, *28*(11), 602–612.

https://doi.org/10.1089/apc.2014.0193

d'Ettorre, G., Ceccarelli, G., Giustini, N., Serafino, S., Calantone, N., De Girolamo, G., … Vullo, V. (2015). Probiotics Reduce Inflammation in Antiretroviral Treated, HIV-Infected Individuals: Results of the "Probio-HIV" Clinical Trial. *PloS One*, *10*(9), e0137200. https://doi.org/10.1371/journal.pone.0137200

de Carneri, I., Di Matteo, L., & Tedla, S. (1992). A comparison of helminth infections in urban and rural areas of Addis Ababa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *86*(5), 540–541.

Deeks, S. G. (2011). HIV Infection, Inflammation, Immunosenescence, and Aging. *Annual Review of Medicine*, *62*(1), 141–155. https://doi.org/10.1146/annurev-med-042909-093756

DiMeglio, L. A., Wang, J., Siberry, G. K., Miller, T. L., Geffner, M. E., Hazra, R., … Jacobson, D. L. (2013). Bone mineral density in children and adolescents with perinatal HIV infection: *AIDS*, *27*(2), 211–220. https://doi.org/10.1097/QAD.0b013e32835a9b80

Dimock, D., Thomas, V., Cushing, A., Purdy, J. B., Worrell, C., Kopp, J. B., … Hadigan, C. (2011). Longitudinal assessment of metabolic abnormalities in adolescents and young adults with HIV-infection acquired perinatally or in early childhood. *Metabolism*, *60*(6), 874–880. https://doi.org/10.1016/j.metabol.2010.08.007

Falasca, K., Vecchiet, J., Ucciferri, C., Di Nicola, M., D'Angelo, C., & Reale, M. (2015). Effect of Probiotic Supplement on Cytokine Levels in HIV-Infected Individuals: A Preliminary Study. *Nutrients*, *7*(10), 8335–8347. https://doi.org/10.3390/nu7105396

George, P. J., Kumar, N. P., Sridhar, R., Hanna, L. E., Nair, D., Banurekha, V. V., … Babu, S. (2014). Coincident Helminth Infection Modulates Systemic Inflammation and Immune Activation in Active Pulmonary Tuberculosis. *PLoS Neglected Tropical Diseases*, *8*(11). https://doi.org/10.1371/journal.pntd.0003289

Harris, P. A., Taylor, R., Thielke, R., Payne, J., Gonzalez, N., & Conde, J. G. (2009). Research electronic data capture (REDCap)—A metadata-driven methodology and workflow process for providing translational research informatics support. *Journal of Biomedical Informatics*, *42*(2), 377–381. https://doi.org/10.1016/j.jbi.2008.08.010

Hunt, P. W., Sinclair, E., Rodriguez, B., Shive, C., Clagett, B., Funderburg, N., … Lederman, M. M. (2014). Gut Epithelial Barrier Dysfunction and Innate Immune Activation Predict Mortality in Treated HIV Infection. *Journal of Infectious Diseases*, *210*(8), 1228–1238. https://doi.org/10.1093/infdis/jiu238

Jiang, M., Deng, K., Jiang, C., Fu, M., Guo, C., Wang, X., … Xin, H. (2016). Evaluation of the Antioxidative, Antibacterial, and Anti-Inflammatory Effects of the *Aloe* Fermentation Supernatant Containing *Lactobacillus plantarum* HM218749.1. *Mediators of Inflammation*, *2016*, 1–8. https://doi.org/10.1155/2016/2945650

Kalinkovich, A. & Weisman, Z & Greenberg, Z & Nahmias, J & Eitan, S & Stein, M. & Bentwich, Zvi. (1999). Decreased CD4 and increased CD8 counts with T cell activation is associated with chronic helminth infection. *Clinical and experimental immunology*. 114. 414-21. 10.1046/j.1365-2249.1998.00736.x

Kelesidis, T., Kendall, M. A., Yang, O. O., Hodis, H. N., & Currier, J. S. (2012). Biomarkers of microbial translocation and macrophage activation: association with progression of subclinical atherosclerosis in HIV-1 infection. *The Journal of Infectious Diseases*, *206*(10), 1558–1567. https://doi.org/10.1093/infdis/jis545

Kent, K., Harper, W., & Bomser, J. (2003). Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells. *Toxicology in Vitro*, *17*(1), 27–33.

Klatt, N. R., Funderburg, N. T., & Brenchley, J. M. (2013). Microbial translocation, immune activation, and HIV disease. *Trends in Microbiology*, *21*(1), 6–13. https://doi.org/10.1016/j.tim.2012.09.001

Kohler, S., & Thiel, A. (2009). Life after the thymus: CD31+ and CD31- human naive CD4+ T-cell subsets. *Blood*, *113*(4), 769–774. https://doi.org/10.1182/blood-2008-02-139154

Krissansen, G. W. (2007). Emerging health properties of whey proteins and their clinical implications. *Journal of the American College of Nutrition*, *26*(6), 713S-23S.

Kundu, C. K., Samanta, M., Sarkar, M., Bhattacharyya, S., & Chatterjee, S. (2012). Food Supplementation as an Incentive to Improve Pre-antiretroviral Therapy Clinic Adherence in HIV-Positive Children--Experience from Eastern India. *Journal of Tropical Pediatrics*, *58*(1), 31–37. https://doi.org/10.1093/tropej/fmr026

Lainka, E., Oezbek, S., Falck, M., Ndagijimana, J., & Niehues, T. (2002). Marked dyslipidemia in human immunodeficiency virus-infected children on protease inhibitor-containing antiretroviral therapy. *Pediatrics*, *110*(5), e56.

Langemead, L, Feakins, R.M., Goldthorpe, S., Holt, H., Tsironi, E., De silva, A., … Rampton, D.S. (2004). Randomized, double-blind, placebo-controlled trial of oral aloe vera gel for active ulcerative colitis - Langmead - 2004 - Alimentary Pharmacology & Therapeutics - Wiley Online Library. *Alimentary Pharmacology & Therapeutics*, *19*(7), 739–747.

Levy, Y., Sereti, I., Tambussi, G., Routy, J. P., Lelievre, J. D., Delfraissy, J. F., …

Lederman, M. M. (2012). Effects of Recombinant Human Interleukin 7 on T-Cell Recovery and Thymic Output in HIV-Infected Patients Receiving Antiretroviral Therapy: Results of a Phase I/IIa Randomized, Placebo-Controlled, Multicenter Study. *Clinical Infectious Diseases*, *55*(2), 291–300. https://doi.org/10.1093/cid/cis383

McIntosh, W. A., Shifflett, P. A., & Picou, J. S. (1989). Social Support, Stressful Events, Strain, Dietary Intake, and the Elderly. *Medical Care*, *27*(2), 140–153.

Means, A. R., Burns, P., Sinclair, D., & Walson, J. L. (2016). Antihelminthics in helminth-endemic areas: effects on HIV disease progression. *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.CD006419.pub4

Miller, T., Borkowsky, W., DiMeglio, L., Dooley, L., Geffner, M., Hazra, R., … Pediatric HIV/AIDS Cohort Study (PHACS). (2012). Metabolic abnormalities and viral replication are associated with biomarkers of vascular dysfunction in HIV-infected children: Vascular dysfunction in paediatric HIV infection. *HIV Medicine*, *13*(5), 264–275. https://doi.org/10.1111/j.1468-1293.2011.00970.x

Olatunya, O. S., Olatunya, A. M., Anyabolu, H. C., Adejuyigbe, E. A., & Oyelami, O. A. (2012). Preliminary Trial of Aloe Vera Gruel on HIV Infection. *The Journal of Alternative and Complementary Medicine*, *18*(9), 850–853. https://doi.org/10.1089/acm.2010.0735

Parakkal, J., Rajamanickam, A., Kumar, N., Kumaraswami, V., Nutman, T., & Babu, S. (2012). Evidence of Microbial Translocation Associated with Perturbations in T Cell and Antigen-Presenting Cell Homeostasis in Hookworm Infections. *PLoS Neglected Tropical Diseases*, *6*, e1830. https://doi.org/10.1371/journal.pntd.0001830

Prendergast, A. J., Chasekwa, B., Rukobo, S., Govha, M., Mutasa, K., Ntozini, R., & Humphrey, J. H. (2017). Intestinal Damage and Inflammatory Biomarkers in Human Immunodeficiency Virus (HIV)-Exposed and HIV-Infected Zimbabwean Infants. *The Journal of Infectious Diseases*, *216*(6), 651–661. https://doi.org/10.1093/infdis/jix367

Rajamanickam, A., Munisankar, S., Bhootra, Y., Dolla, C., Nutman, T. B., & Babu, S. (2017). Microbial Translocation Associated with an Acute-Phase Response and Elevations in MMP-1, HO-1, and Proinflammatory Cytokines in Strongyloides stercoralis Infection. *Infection and Immunity*, *85*(1), e00772-16. https://doi.org/10.1128/IAI.00772-16

Sangare, L.R., Herrin, B. R., John-Stewart, G., & Walson, J. L. (2011). Species-specific treatment effects of helminth/HIV-1 co-infection: a systematic review and meta-analysis. *Parasitology*, *138*(12), 1546–1558.

https://doi.org/10.1017/S0031182011000357

Sprong, R. C., Schonewille, A. J., & van der Meer, R. (2010). Dietary cheese whey protein protects rats against mild dextran sulfate sodium–induced colitis: Role of mucin and microbiota. *Journal of Dairy Science*, *93*(4), 1364–1371. https://doi.org/10.3168/jds.2009-2397

Taylor-Robinson, D. C., Maayan, N., Soares-Weiser, K., Donegan, S., & Garner, P. (2015). Deworming drugs for soil-transmitted intestinal worms in children: effects on nutritional indicators, haemoglobin, and school performance. In *The Cochrane Library*. John Wiley & Sons, Ltd. https://doi.org/10.1002/14651858.CD000371.pub6

van Servellen, G., & Lombardi, E. (2005). Supportive Relationships and Medication Adherence in HIV-Infected, Low-Income Latinos. *Western Journal of Nursing Research*, *27*(8), 1023–1039. https://doi.org/10.1177/0193945905279446

Villar-García, J., Hernández, J. J., Güerri-Fernández, R., González, A., Lerma, E., Guelar, A., … Knobel Freud, H. (2015). Effect of probiotics (Saccharomyces boulardii) on microbial translocation and inflammation in HIV-treated patients: a double-blind, randomized, placebo-controlled trial. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *68*(3), 256–263. https://doi.org/10.1097/QAI.0000000000000468

Wallet, M. A., Rodriguez, C. A., Yin, L., Saporta, S., Chinratanapisit, S., Hou, W., … Goodenow, M. M. (2010). Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T-cell activation following therapy: *AIDS*, *24*(9), 1281–1290. https://doi.org/10.1097/QAD.0b013e328339e228

Walson, J. L., Herrin, B. R., & John-Stewart, G. (2009). Deworming helminth co-infected individuals for delaying HIV disease progression. In The Cochrane Collaboration (Ed.), *Cochrane Database of Systematic Reviews*. Chichester, UK: John Wiley & Sons, Ltd. Retrieved from http://doi.wiley.com/10.1002/14651858.CD006419.pub3

WHO. (2017). Preventive chemotherapy to control soil-transmitted helminth infections in at-risk population groups-Guideline. WHO.

Yang, O. O., Kelesidis, T., Cordova, R., & Khanlou, H. (2014). Immunomodulation of antiretroviral drug-suppressed chronic HIV-1 infection in an oral probiotic double-blind placebo-controlled trial. *AIDS Research and Human Retroviruses*, *30*(10), 988–995. https://doi.org/10.1089/AID.2014.0181

Zimmermann, K., Bastidas, S., Knecht, L., Kuster, H., Vavricka, S. R., Günthard, H. F., & Oxenius, A. (2015). Gut commensal microbes do not represent a dominant antigenic source for continuous CD4+ T-cell activation during HIV-1 infection.

European Journal of Immunology, *45*(11), 3107–3113.
https://doi.org/10.1002/eji.201545940

CHAPTER 5

Final Thoughts and Future Directions

The data collected through this trial and the information gained has contributed to the understanding of pediatric HIV in a developing world context. The impacts of growth failure and high viral loads on T-cell distribution have not been well explored but our findings would indicate they both have implications for pediatric HIV. The findings from this trial definitely support the development of further trials to explore associations between growth failure, virologic failure and distribution of T-cell subsets. Also, the reported decreases in levels of sCD14 along with the significant increases in the proliferating naïve CD4 subset coupled with significant growth gains in this cohort warrants further investigation. Our tentative conclusions that antihelminths and social support may have contributed to these findings should be explored more systematically. Assessing leptin and growth hormone levels in this cohort would be very interesting in light of the significant growth failure that we documented and the known associations these hormones have with metabolism (Cohen, Danzaki, & MacIver, 2017; Holländer, Krenger, & Blazar, 2010; Welniak, Sun, & Murphy, 2002).

Strengths and Limitations

The longitudinal design of this trial and its focus on evaluating interventions to address chronic immune activation in pediatric HIV was a strength. Conducting this trial in Ethiopia was a challenge but a strength in that capturing data that is relevant to pediatric HIV care in a developing country is a positive contribution to the scientific community and to health care. The large majority of the approximately 2.1 million children living with HIV worldwide live in developing countries where environments are

very different than in western countries (Abel, 2009). The exposure this trial allowed for clinic staff to clinical research was a positive contribution. The use of an iso-caloric comparison group helped to identify contributing factors to our results and the palatable flavored drinks was a strength which likely helped to support the high compliance in this trial. The use of the REDCap database and tracking system through MSU's Bioinformatics Research Informatics Core (BRIC) allowed for continuous data entry and monitoring throughout the duration of this trial and for rapid compilation and transfer of data into SPSS and JMP software programs.

The lack of a comparison group of HIV- children was a weakness. Since little is known about T-cell distributions or immune activation in Ethiopian children, a control group would have allowed us to better understand our findings. The lack of a marker to monitor compliance of daily intake was another limitation. Given the culture of Ethiopia were sharing is the norm, it is very possible that the daily supplement was shared between family members which would have diluted the effect on the enrolled child. Also, providing antihelminths to all participants did not allow an assessment of the impact of this intervention separate from the nutritional support interventions. The relatively small sample sizes and short duration of this trial were also limitations, however as a pilot trial with limited funding a longer trial was not feasible.

There is limited research that has been done in pediatric HIV populations making the selection of appropriate biomarkers and interpretation of results more challenging. This led to our using both planned and exploratory approaches in data analysis. In evaluating large numbers of variables together it is recommended to lower levels of statistical significance to avoid reporting of levels of significance that may occur by

chance (type 1 errors). Unfortunately, when conducting exploratory trials like this was, lowering the significance level too low can also obliterate significant associations that have biological relevance (type II errors). In this trial we chose to not adjust for multiple comparisons. The number of associations we noted between variables related to interventions in both the control and aloe/whey groups are unlikely to be due to chance alone.

Next Steps

While the clinical trial has been completed and much of the analysis done there are still parts to this project that need to be completed. We plan to enroll a small cohort of healthy age matched children in the near future to allow for clearer determinations of study findings especially in regard to the distribution of T-cell subsets in health Ethiopian children. We also plan to assess inflammatory markers (IL-1β, IL-6 and TNFα), as well as markers of antioxidant status on stored plasma samples for this cohort. These assays were part of the original protocol design but were put on hold due to complications in kit acquisition and time constraints. The results of these assays will also provide further information about this cohort of HIV children, especially in regard to the levels of sCD14 that we detected as the presence of sCD14 has been associated with markers of inflammation.

Early diagnosis and initiation of ART has turned the prognosis and clinical outcomes of pediatric HIV in a much more favorable direction. Unfortunately, there is still much to learn and implement in regards to1the growing awareness of chronic immune activation, inflammation and the impacts on the development of co-morbid diseases in pediatric HIV populations. Identifying and evaluating effective, safe,

affordable and sustainable interventions to attenuate chronic immune activation in HIV is a research priority. While the nutritional interventions we trialed did not show clear impacts on measures of microbial translocation, the exploration of other nutritional interventions such as probiotics is warranted and of interest.

REFERENCES

REFERENCES

Abel, K. (2009). The rhesus macaque pediatric SIV infection model - a valuable tool in understanding infant HIV-1 pathogenesis and for designing pediatric HIV-1 prevention strategies. *Current HIV Research*, *7*(1), 2–11.

Cohen, S., Danzaki, K., & MacIver, N. J. (2017). Nutritional effects on T-cell immunometabolism. *European Journal of Immunology*, *47*(2), 225–235. https://doi.org/10.1002/eji.201646423

Holländer, G. A., Krenger, W., & Blazar, B. R. (2010). Emerging strategies to boost thymic function. *Current Opinion in Pharmacology*, *10*(4), 443–453. https://doi.org/10.1016/j.coph.2010.04.008

Welniak, L. A., Sun, R., & Murphy, W. J. (2002). The role of growth hormone in T-cell development and reconstitution. *Journal of Leukocyte Biology*, *71*(3), 381–387.