DIRECT BIOMARKERS OF MICROBIAL TRANSLOCATION AS PREDICTORS OF IMMUNE ACTIVATION IN ADULT ZAMBIANS WITH ENVIRONMENTAL ENTEROPATHY AND HEPATOSPLENIC SCHISTOSOMIASIS

By

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DEDICATION

I dedicate this thesis to my wife, Sylvia Kaonga and my children Kondwani, Lombani and Taonga for all the support and love during the course of this journey even when I robbed them of the family time.

To my legendary mother and late father they taught me to work hard, perseverance and dedication.

To my brother and sisters, thank you for all the support, encouragements and love that I continue to receive over the years.

Without you all, this work would not have been possible.

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DECLARATION

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APPROVAL

This PhD thesis by **Patrick Kaonga** is approved in fulfillment of the requirements for the award of the Doctor of Philosophy in Immunology by the University of Zambia.

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ABSTRACT

Background: Microbial translocation is a poorly understood consequence of several disorders such as environmental enteropathy (EE) and hepatosplenic schistosomiasis (HSS) disease. Direct biomarkers of microbial translocation such lipopolysaccharide, 16S rRNA gene and Toll-like receptor ligands may predict immune activation. This study evaluated whether direct biomarkers of microbial translocation correlate and predict immune activation in adult Zambians with EE and HSS disease. The public health importance of biomarkers is that they can be used to predict individuals with EE or HSS who are likely to develop chronic immune activation or are at risk. The biomarkers may be used for early diagnosis and stratify for any treatment or intervention.

Methods: An unmatched case-control study was conducted in participants with EE (n=67) recruited from Misisi compound, Lusaka, Zambia, with two comparison groups, HSS participants (n=86) from The University Teaching Hospital and healthy controls (n=41). Plasma lipopolysaccharide (LPS) was measured by Limulus Amoebocyte Lysate Assay, plasma 16S rRNA gene copy number was quantified by quantitative real-time PCR, Toll-like receptor ligands (TLRLs) activity by QUANTI-Blue detection medium, plasma biomarkers of host response (C-reactive protein, soluble CD14, soluble CD163 and lipopolysaccharide-binding protein) to microbial translocation were measured by ELISA and cytokines (TNF-α, IL-6, IL-10, IL-4, IL-2, IFN-γ and IL-17) from cell culture supernatant by Cytometric Bead Array.

Results: Plasma lipopolysaccharide levels were elevated in EE group with median 378.9 (IQR, 82.7 - 879.5) EU/ml compared to participants with HSS with median 213.1 (IQR, 77.2 - 358.3) EU/ml; p=0.03 or healthy controls with median 202.3 (IQR, 43.2 - 251.1) EU/ml; p=0.01. The 16S rRNA copy number were significantly elevated in the EE group with median 2651 (IQR, 529 – 8779) copies/µl compared to the levels in participants with HSS with median 387 (IQR, 165 – 1990) copies/µl; p<0.001) or healthy controls with median 193 (IQR, 132 – 455) copies/µl; p<0.001. TLRLs activity was significantly higher in the EE group with median 0.49 (IQR, 0.0 - 0.8) OD units than in participants with HSS with median 0.13 (IQR, 0.0 - 0.8) OD units; p=0.01 or the healthy controls with median 0.02 (IQR, 0.0 - 0.12) OD units; p=0.004. Participants with HSS had higher TLRLs activity compared to healthy controls p=0.02. In multivariate multiple regression models LPS, 16S rRNA copy number, and TLRL activity were independent predictors of cytokines while controlling for baseline characteristics. In the EE group, a good model fit was obtained ($R^2 = 0.526$, $F = 47.53$, $p < 0.001$) which predicted TNF-α, IL-6, and IL-10. In the HSS group, a less impressive but still significant fit was obtained ($R^2 = 0.382$, $F = 22.43$, $p = 0.002$ which predicted 16S rRNA and TLRLs. In healthy controls, no satisfactory model was obtained ($R^2 = 0.040$, $F = 1.03$, $p = 0.38$).

Conclusions: Direct biomarkers of microbial translocation were higher in EE and HSS participants compared to healthy controls. The biomarkers seems to correlate and predict immune activation in individuals with EE and HSS infection. This data support the model that proposes that biomarkers of microbial origin in the gastrointestinal tract move across a

compromised intestinal barrier leading to heightened immune activation in conditions with intestinal barrier dysfunction. The study recommends measurement of microbial translocation using these biomarkers. They are cheaper, practical and non-invasive.

Key words: Microbial translocation, Biomarkers, Environmental Enteropathy, Hepatosplenic Schistosomiasis

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ABBREVIATIONS AND ACRONYMS

CBA	Cytometric Bead Array
CD	Cluster of Differentiation
CD163	Cluster of Differentiation 163
CpG-DNA	Cytosine-phosphate-guanosine deoxyribonucleic acid
CRP	C-Reactive Protein
EE	Environmental Enteropathy
ELISA	Enyzme-Linked Immunosorbant Assay
FACS	Fluorescence-Activated Cell Sorter
FBS	Fetal Bovine Serum
HD	Human defensin
HIV	Human Immunodeficiency Virus
HSS	Hepatosplenic Schistosomiasis
IBDs	Inflammatory Bowel Diseases
IFN-γ	Interferon-gamma
IL	Interleukin
LAL	Limulus Amoebocyte Lysate
LBP	Lipopolysaccharide-Binding Protein
L:M	Lactulose Mannitol ratio
LPS	Lipopolysaccharide
MLNs	Mesenteric Lymph Nodes

MT	Microbial Translocation
MyD88	Myeloid Differentiation Factor 88
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PRR	Pathogen Recognition Receptor
sCD14	Soluble Cluster of Differentiation 14
sCD163	Soluble Cluster of Differentiation 163
SEAP	Secreted Embroyonic Activated Phosphatase
TLRL	Toll-like Receptor Ligand
TNF-α	Tumor Necrosis Factor-alpha
UNZA	University of Zambia
UNZABREC	University of Zambia Biomedical Research Ethics Committee
16S rRNA	16 subunit Ribosomal Ribonucleic Acid

DEFINATION OF TERMS

Microbial translocation is the movement of microbes or microbial products from the gastrointestinal tract into the mesenteric lymph nodes and other sterile sites such blood circulatory system (Kelly et al., 2015).

Biomarkers is a molecule which can be quantified and by which a particular condition or pathological process or disease can be identified (Biomarker Definition Working Group, 2001).

Environmental enteropathy is an asymptomatic condition hypothesized to be caused by constant fecal-oral contamination with subsequent blunting of intestinal villi and small intestinal inflammation (Korpe and Petri, 2012).

Hepatosplenic schistosomiasis refers to the main complication of chronic infection caused by a number of Schistosoma species including but not limited to *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi* and *Schistosoma intercalatum*. HSS denotes a distinct mode of intrahepatic portal hypertension characterised by a spared liver parenchyma and a partly blocked portal vein. (Sharker *et al*., 2014; Marinho 2010).

Toll-like receptors are a type of Pathogen Recognition Receptors (PRR) that occur as membrane proteins expressed by almost all

mammalian cells. They typically contain 10 domains comprised of leucine-rich repeats (LRRs) that are found on the cells of innate immune system such as macrophages and dendritic cells. These recognize structurally conserved molecules of microbes (Janssens and Beyaert, 2003).

Lipopolysaccharide	is a major component of the Gram-negative cell wall, highly antigenic and a ligand for TLR4. Since humans do not have LPS, its presence in blood is considered as a direct biomarker of microbial translocation (Marchetti *et al*., 2013; Fukui, 2016).
16S rRNA	is the component of the 30S small subunit of the prokaryotic ribosome that binds to the shine-Dalgarno sequence (Woese and Fox, 1977).
CD163	a biomarker of Kupffer cell activation, and a macrophage secreted as sCD163 which is upregulated in inflammatory diseases (Jone *et al*., 2013).
Soluble CD14	is a target for a complex of LPS-LBP and an indirect biomarker of microbial translocation. In the presence of LPS, the expression of sCD14 is upregulated and act together with MD2 and TLR4 to initiate clearance of LPS (Koutsounas *et al*., 2015).
LBP	is a soluble acute-phase protein that binds bacterial LPS and interacts with CD14 and myeloid differentiation

factor 2 (MD-2). This complex binds to Toll-like receptor 4 (TLR4) to initiate an immune activation (Muta and Takeshige, 2001).

CHAPTER ONE: INTRODUCTION

1.0 Background

Microbial translocation (MT) is the passive movement of microbes or their products from the gut into mesenteric lymph nodes and other sterile sites. It is common in disorders with intestinal barrier dysfunction such environmental enteropathy (Kelly *et al*., 2015) and hepatosplenic schistosomiasis (Sinkala *et al*., 2015). It is also a major driver of mortality in critical care settings throughout the world, and complicates several surgical disorders of the abdomen including intestinal obstruction and volvulus. MT can be measured by detection of direct biomarkers in plasma such as lipopolysaccharide (LPS), 16S ribosomal RNA (16S rRNA) gene copy number, or other Pathogen-Associated Molecular Patterns (PAMPs). It has also been measured indirectly through host response biomarkers, though this is a less ‘pure’ approach which could be influenced by other disease processes. Previous studies have measured direct biomarkers of MT using LPS (Jiang *et al*., 2009) and 16S rRNA gene copy number without detecting other microbial components which could lead to chronic immune activation and if not controlled may results into microcirculatory dysfunction, septic shock, tissue damage, and mortality (Mocellin *et al*., 2002).

It has been known for a long time that people living in areas of poor sanitation and hygiene have a widespread phenomenon of asymptomatic abnormal structure and functional changes in the small intestine (Korpe and Petri, 2012). The first description of the functional disorder of the small intestine was reported by William Hillary in the 1700s when he observed in some European expatriates working in Barbados. In the 1800s Patrick Manson a Scottish physician noted similar changes in some Dutch people from the West Indies. Some reports of abnormal structure and

functional changes of the small intestine followed in the 1900s from Europeans in Southern Asia during the Second World War. Much later in the 1960s, some structural and functional changes were documented in residents of some tropical countries and American Peace Corps Volunteers deployed in India and Pakistan for a few years (Lindenbaum *et al.*, 1971). Small intestine abnormal microscopic changes were also noticed in adult Indians in the early 1960s.

The affected individuals appeared healthy, which partly explains why the health implications of this condition have not received much public attention (Watanabe and Petri, 2016). The condition is characterised by blunted and shortened villi with increased intraepithelial lymphocytes leading to reduced absorption surface area (Korpe and Petri, 2012; Prendergast and Kelly, 2012), which other studies have also demonstrated in biopsies of small intestines from adults in many developing countries. The results showed some consistent findings of pathological changes, including but not limited to decrease in villus height, crypt depth and lymphocytic infiltration (Kelly *et al.*, 2004; Denno *et al.*, 2010; Louis-Auguste *et al.*, 2014; Mannary *et al.*, 2010).

The magnitude of the problem in the early days was not known and all previous studies were in adults. Although the cause of the problem is still elusive, at that time, the condition was thought to affect children as well. The phenomenon was referred to as ‘tropical enteropathy' because it was believed to exist in tropical countries. Later more studies emerged and showed that the environment had a bigger role to play than climate (Menzies *et al.*, 1999). Tropical enteropathy was renamed ‘environmental enteropathy' (Brunser *et al.*, 1990) as it became clear that tropical countries were not the only ones affected by this condition.

Conversely, not all tropical countries have reported cases of environmental enteropathy (EE): Qatar and Singapore are two examples (Menzies *et al.*, 1999). The condition, however, appears to depend on exposure to certain environmental conditions such as faecal-oral contamination (Brunser *et al*., 1990). In the beginning, treatment was made based on different methods used such as the use of antibiotics, antiparasitics and micronutrient response after pragmatic treatment. However, intervention to which individuals responded to remained unclear (Crane *et al*., 2015).

Currently, diagnosis of the EE depends partly on endoscopy and histological demonstration of abnormalities in the small intestine and permeability of the small intestine measured by sugar tests such as lactulose and mannitol (L:M) ratio. Many of the studies were carried out in adults. To date, the description and significant efforts to understand EE have moved towards intestinal dysfunction observed in the affected individuals in an attempt to understand better and emphasise that EE is not due to climate, genetic or other factors as earlier thought. The functional changes in the small intestine, such as permeability are easier to measure compared to the histological changes referred to as "enteropathy", so in recent years there has been some impetus to renaming the disorder "Environmental Enteric Dysfunction" (EED) (Keusch *et al*., 2014). The term currently is preferred by some workers because there is no association between the condition and clinical symptoms. The new name is meant to focus on impaired intestinal function (McKay *et al*., 2010). However, the term EED ignores the histological manifestations of the disorder. It must be highlighted that EE is not a disease but a change that is noticed in a population with inadequate water supply and sanitation which predisposes individuals to microbial overload through the faecal-oral route and ultimately lead to MT (Humphrey, 2009; Campbell, 2003; Kosek *et al*., 2014). In this study, the term environmental enteropathy (EE) is used because to confirm enteropathy, individuals; underwent endoscopy (to detect structural changes) as opposed to function tests.

Chronic infection of parasites such as *Schistosoma mansoni, Schistosoma japonicum*, *Schistosoma intercalatum* and *Schistosoma mekongi* may lead to hepotosplenic schistosomiasis (HSS) disease (Jia *et al*., 2011). HSS is associated with morbidity and mortality due to oesophageal and gastric varices which are formed and later bleed as a result of portal hypertension which is one of the commonest consequence of HSS (Shaker *et al*., 2014; Chofle *et al*., 2014). When oesophageal and gastric varices bleeds, the sites may be possible routes for microbes in the gut to move from the intestine to the mesenteric lymph nodes and/or portal vein and later into the systemic circulation (Bellot *et al.*, 2013; Wiest *et al*., 1999; Balzan *et al*., 2007).

This study was set out to compare three different approaches to measurement of MT in blood samples, by measuring LPS, bacterial DNA (16S rRNA), and Toll-like receptor ligands (TLRLs) activity, which detects almost all possible Pathogens Associated Molecular patterns (PAMPs) in plasma. Furthermore, to determine whether direct biomarkers are predictors of immune activation in individuals with EE and HSS.

1.2 Statement of the Problem

Studies have shown that only LPS and 16S rRNA gene (16S rRNA) have been used as direct biomarkers of MT and are immune stimulants which can lead to chronic immune activation (Jiang *et al*, 2009; Brenchley *et al*., 2006). These do not, however, represent all possible PAMPs. LPS is only present in Gram-negative bacteria, and 16S rRNA is only present in bacteria, and these exclude other PAMPs components from bacteria, fungi, and viruses which could act as immune stimulants. Other PAMPs include lipopeptides, peptidoglycan, lipoteichoic acid, double-stranded RNA, flagella, single-stranded RNA and unmethylated CpG DNA which currently not measured by using common assays such as ELISA and PCR. By using reporter cell lines such as RAW-Blue

mouse macrophage cell line, it is possible to detect all possible PAMPs in plasma which gives the total PAMPs read out responsible for immune stimulation. When the body is exposed to PAMPs (microbial origin), it responds to these PAMPs producing biomarker that are considered host response to microbial translocation such as sCD14, sCD163, lipopolysaccharide-binding protein (LBP) and C-reactive protein (CRP) among others.

The relationship between host immune response and direct biomarkers of microbial translocation has not been adequately established in individuals with EE and HSS. Also, no study has compared levels of these biomarkers in both EE and HSS patients which are common conditions in Zambia and an individual with both conditions the effect could probably be more detrimental. MT is difficult to measure, there is no single biomarker and no specific biomarker. The hallmark of MT is chronic immune activation, which could lead to the establishment of disease in the intestinal mucosa resulting in many consequences. These include microcirculatory dysfunction, induction of intravascular coagulation (Ebersoldt *et al*., 2007) hemodynamic disturbances, metabolic derangements (Levi and Ten Cate, 1999) suppression of cellular immunity, septic shock, and death (Liu and Malik, 2006).

1.3 Justification of the Study

In past few years, there have been some studies to explore and understand biomarkers of MT in EE both in human (Manary *et al*., 2010; Kelly *et al*., 2016) and animal models (Brown *et al*., 2015). To date, attention has focused mainly on measuring LPS and 16S rRNA in plasma which both indicate the presence of bacteria. In HSS patients the only direct biomarker that has been used is LPS while surrogate biomarkers such as CRP, LBP, sCD14, and sCD163 (Sinkala *et al*., 2016) have also been used. These direct biomarkers are not the only immune stimulators of microbial

origin. Other microbes such as fungi and viruses also have the potential to cause immune activation together with other bacterial components. In this regard, less attention has been given to other possible immune stimulators, and this warrants a comprehensive analysis of all possible immune stimulators of microbial origin (PAMPs). The result could lead to the development of improved management of individuals with EE and HSS patients and potential to deliver much-needed robust evaluation of direct biomarkers to permit assessment of interventions. Currently, there is no data on the use of 3 different approaches LPS, 16S rRNA and TLRL activity to measure microbial translocation and information whether these can be immune activation predictors in both EE and HSS patients. There is also no information indicating which one of these is the highest predictor of immune activation in EE and HSS patients.

Many studies have demonstrated MT by detecting some direct biomarkers (LPS and 16S rRNA) in plasma in both human and animal models. Currently, there is no data on the detection of total PAMPs (TLRL) activity in individuals with EE and HSS patients and comparison of these biomarkers has never been done in those two groups. Possible PAMPs that can stimulate immune activation include lipopeptides, peptidoglycan, lipoteichoic acid, double-stranded RNA (ssRNA), flagella, single-stranded RNA and unmethylated CpG DNA. The mechanisms how direct biomarkers cross the intestinal barrier into the systemic circulation has been proposed to be through the leaky pathway which has been partly elucidated (Turner *et al*., 2009). Many in vitro experimental studies have demonstrated that LPS-stimulated RAW-Blue mouse macrophages cells produce a number of both pro-inflammatory and anti-inflammatory cytokines (Park, 2014; Yoon *et al*., 2016; Soromou *et al*., 2013). RAW-Blue macrophage cell line stably expresses a secreted embryonic alkaline phosphatase (SEAP) inducible by a transcription factor. They express all Toll-

like receptors (TLRs) except TLR5 which when stimulated by PAMPs lead to the secretion of SEAP which is detectable by detection medium, QUANTI-Blue, indicating the total PAMPs (TLRL) activity which can be quantified. Plasma-stimulated RAW-Blue mouse macrophage cell line could give total PAMPs activity readout which is useful in quantification of TLRL activity in both individuals with EE and HSS patients which could indicate the presence of direct biomarkers in plasma and this could potential be useful in early detection of microbial translocation. The proposed mechanisms of EE and HSS is shown in Figure 1.1.

HSS

Repeated contact with infected water bodies by S. mansoni, S. japonicum, S. intercalatum and S. mekongi (Payne *et al*., 2013; Mugono *et al*., 2014)

Chronic infection for years results in HSS (Da Silva *et al*., 2005; Mazingo *et al*., 2015)

Development of: Periportal fibrosis, increased portal hypertension, Oesophageal varices and gastric varices (Wynn *et al*., 2004; Aggarwal *et al*., 2014)

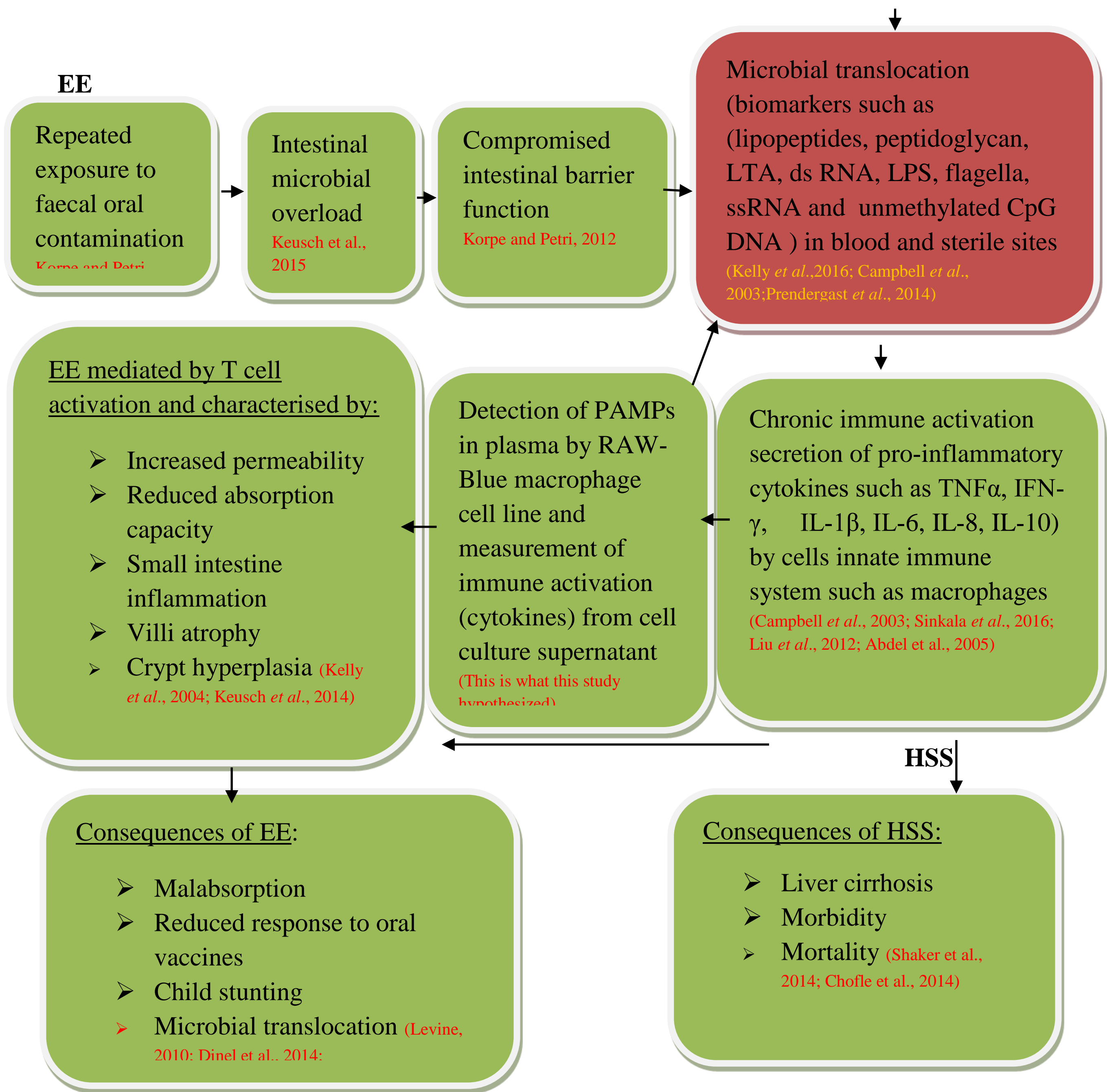


Figure 1.1: Conceptual framework showing hypothesised cause and consequences of EE and HSS due to repeated exposure to faecal oral contamination and schistosomal species infected water bodies respectively. Both situations may lead to microbial translocation. It is envisaged that using RAW-Blue macrophage cell line can detect PAMPs that contribute to immune activation.

1.4 Research Question

To what extent do direct biomarkers of microbial translocation predict and correlate immune activation in individuals with environmental enteropathy and hepatosplenic schistosomiasis?

1.5 General Objective

To assess whether direct biomarkers of microbial translocation predict immune activation in individuals with environmental enteropathy and hepatosplenic schistosomiasis.

1.6 Specific Objectives

1. To quantify direct biomarkers of microbial translocation in individuals with environmental enteropathy, hepatosplenic schistosomiasis and healthy controls.

2. To determine host immune responses to microbial translocation in environmental enteropathy, hepatosplenic schistosomiasis and healthy controls.

3. To determine immune activation in individuals with environmental enteropathy, hepatosplenic schistosomiasis and healthy controls using cell culture *in-vitro* model.

4. To determine the relationship among plasma direct biomarkers of microbial translocation and immune activation measured from cell culture supernatant in individuals with environmental enteropathy, hepatosplenic schistosomiasis and healthy controls.

CHAPTER TWO: LITERATURE REVIEW

2.1 Epidemiology of Environmental Enteropathy

EE is a widely distributed but neglected public health problem (Louis-Auguste *et al*., 2014). Early studies showed that it is common in some areas of Asia, Africa and the Caribbean based on intestinal absorption function and permeability of the gut. A geographical study of asymptomatic volunteers in twenty countries was conducted by Menzies and colleagues (1999) in which the integrity of the intestine was assessed using four-sugar tests. These included 3–O-methyl-D-glucose to evaluate effective absorption, D-xylose which assesses passive absorption and carrier-mediated, and non-carrier mediated using L-rhamnose ratio which measures absorptive capacity, as well as intestinal permeability using lactulose (L). The results showed that residents of tropical countries had significantly higher L:M ratio compared with residents of the USA, Europe or Qatar (Menzies *et al*., 1999). They also had higher lactulose: rhamnose ratio indicating higher permeability and lower sugar absorption. The results suggested the presence of EE in tropical countries (Figure 2.1) and not in the USA, Europe, and Qatar. Another interesting finding which was independent of climate was the correlation of Gross Domestic Product (GDP) with the intestinal absorption capacity suggesting that poverty was more important than climate (Menzies *et al*., 1999). In Australian Aboriginal children exposed to environmental hazards similar to those found in developing countries, intestinal permeability was found in about one-third of them compared to non-Aboriginal children (Kukuruzovic *et al*., 2002) suggesting the presence of EE.

In Bangladesh, the average D-xylose absorption for children aged 2-64 months was about 66% of the value in children in the USA, indicating the intestinal dysfunction of the small intestine and suggesting EE (Einstein *et al*., 1972). An observational study in Bangladesh found increased

intestinal dysfunction in children during their first year of life living in urban slums and was associated with malnutrition and diarrheal diseases (Einstein *et al*., 1972). A similar study in Thailand reported about 50% of children living in slums aged 2.5 – 9 years had D-xylose absorption capacity below normal (Colwell *et al*., 1968). In a trial in Bangladesh intestinal mucosal function, as measured by sugar permeability, was impaired among severely underweight children. The investigators also found intestinal permeability improved after weight gain, but intestinal mucosal recovery was not explicitly related to the types or amount of food supplementation or psychosocial stimulation that was provided in the study (Hossain *et al*., 2010). In all these studies it was suggested that EE could be the underlying cause or contributing factor. In Eastern Pakistan mucosal inflammation, blunted villi, with leaf-like morphology were observed in jejunal biopsies of asymptomatic adults fitting the typical description of EE (Lindenbaum *et al*., 1971; Lindenbaum *et al*., 1966).

In Southern India cases of tropical sprue, an infectious disease which responds to antibiotic treatment was common in the past, and people attributed changes in intestinal morphology and function to the disease. Even after the disappearance of the disease variations in the intestinal morphology and function continued to be seen and these were believed to be cases of EE (Rolston and Mathan, 1990). Even much earlier, in India, stillborn foetuses at post-mortem revealed the presence of intestinal morphological changes where villi were observed to have a leaf-like shape as opposed to normal finger-like projections suggesting the presence of EE (Chacko *et al*., 1969). Studies conduted in adult Vietnamese and North Americans compared asymptomatic indigenous individuals who had no history of tropical exposure. Jejunal biopsy samples showed some histological characteristics which were different in the two popution. Biopsy samples from

Vietnamese were infiltrated with immune cells and had, shortened and broadened villi as well as reduced crypt depth but not in the Americans. Further, when the sugar absorption test was performed and compared, it was observed that the abnormalities were only found in the Vietnamese population (Chacko *et al*., 1969). A number of countrires reported similar results in Thailand, Pakistan, Malaysia, and Hong Kong and these findings were characteristic and suggestive of EE (Colwell *et al*., 1968).

In Peru, a study showed that an analysis of children from a region where EE is endemic (Yori *et al*., 2014). Both urine concentration and fraction excretion of lactulose was measured and found significantly higher compared to the USA controls. Even after comparing the lactulose to rhamnose ratio (LRR) similar results were obtained indicating increased intestinal permeability suggesting EE (Faubion *et al*., 2016). In some parts of the world such as West Indies intestinal structure and function were found to be abnormal in most residents. Inflammatory cells and lymphocytes infiltration in the lamina propria were observed in intestinal biopsies also suggesting the presence of EE (Klipstein *et al*., 1968).

To further show that EE is common in settings with poor sanitation and hygiene, asymptomatic Indian and Pakistani immigrants had their xylose absorption tested and jejunal biopsies for histology examination were taken and were both found to be abnormal upon arrival in the USA. Significant improvement toward normal in both xylose absorption and villus architecture occurred with increasing periods of residence in USA (Gerson *et al*., 1971) suggesting environment had a big role to play. In 61 subjects that were studied in Haiti, xylose absorption was found to be abnormally low in all the subjects apart from only a few, but their jejunal morphology was

abnormal for all subjects. However, although it was partly reported to be due to tropical sprue, it is possible that EE was masking the finding since the study was done in a rural community with poor sanitation and hygiene (Klipstein *et al*., 1968). In a Mexican study, both healthy looking children and adults on jejunal mucosa biopsy showed loss of villi, increased cellularity in the lamina propria with moderate flattening and inflammatory infiltration with few glands and crypt observed. The subjects that showed moderate to severe abnormal architecture of the mucosa also had abnormal D-xylose urine excretion (Garcia, 1968) and the findings were consistent with EE as shown in Figure 2.1.

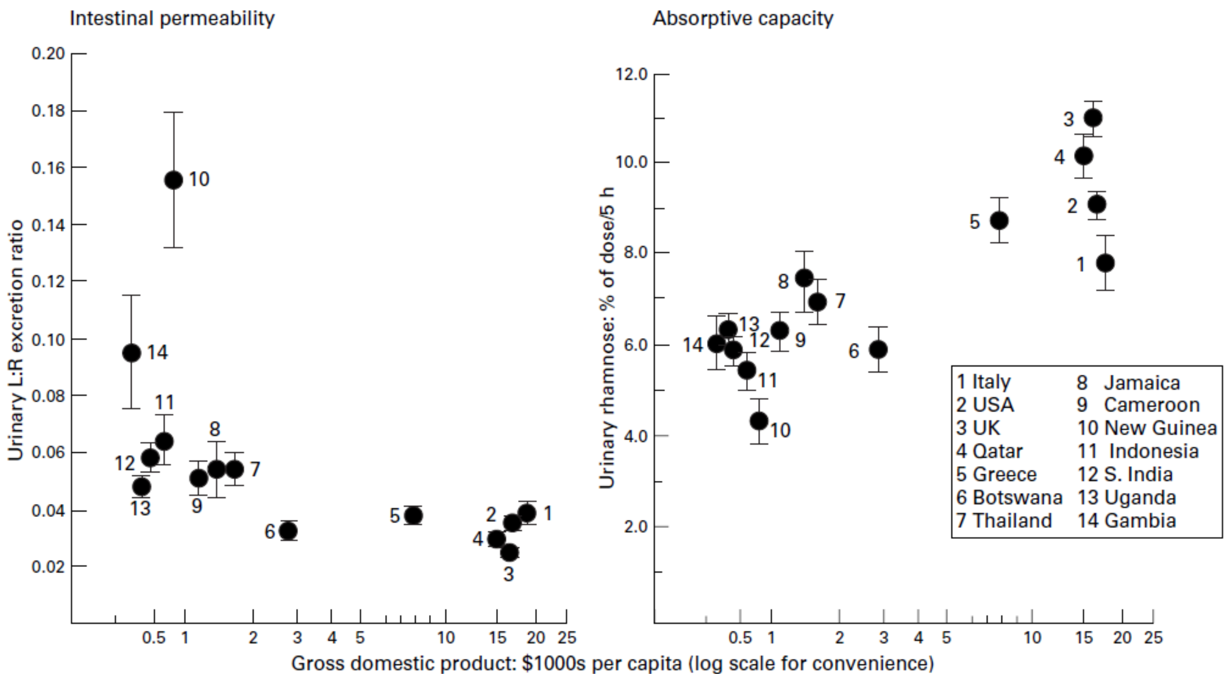


Figure 2.1: Correlation of intestinal permeability and absorption capacity with gross domestic product per capita for country of residence plotted on a log scale (Source: Menzies et al., 1999).

2.2 Environmental Enteropathy in Africa

In Africa, EE is one of the persistent and prevalent conditions linked to abnormal histology and dysfunction of the gut (Weisz *et al*., 2012). It is suggested to be associated with stunting in children

(Prendergast *et al*., 2014) especially in rural settings (Keusch *et al*., 2014). Some studies have demonstrated that EE is prevalent especially in the rural areas in The Gambia. The condition has been reported to be associated with growth faltering in children in the Gambia (Campbell *et al*., 2003). In a cohort study conducted in a rural area of the Gambia, EE was found to be common, and markers of EE were L:M ratio, a marker of intestinal permeability and faecal neopterin, a biomarker of gut inflammation were found to be inversely associated with growth suggesting that children with EE exhibited growth failure. Small intestinal permeability determined by small bowel biopsies and all tested Gambian children had chronic cell-mediated enteropathy with short villi, high inflammatory cell and crypt hyperplasia in the lamina propria when compared to the healthy controls from the United Kingdom (Campbell *et al*., 2003). In the same country, an earlier study in children from another rural area showed that poor childhood growth was associated with small bowel permeability related to EE (Lunn *et al*., 2000). Furthermore, another study in a different rural setting reported that children were characterized by the mucosal inflammatory response and was associated with intestinal permeability. The same investigators also demonstrated that EE is widespread in children living in rural The Gambia. EE was determined by measuring L: M ratio and children with this condition were reported to be stunted. Linear growth of children was associated with persistent, abnormal bowel mucosa and close to half of the children observed with growth faltering were attributed to the abnormal gut mucosa (Lunn *et al*., 1991).

In a recent study, children with EE in different epidemiological surroundings which aimed to explain alterations in intestinal permeability, the results showed that L: M ratio is a poor predictor of growth failure in the Malnutrition and the Consequences for Child Health and Development (MAL-ED) study (Kosek *et al*., 2017). In healthy Sudanese subjects L: M ratio was compared to that of healthy English subjects as a measure of small intestinal permeability. It was reported that

the L: M ratio was significantly higher in Sudanese subjects compared to the English subjects indicating abnormal intestinal permeability suggesting presence EE in the Sudanese (Ukabam *et al*., 1986). In Nigeria, jejunal biopsies taken from apparently normal healthy adults and assessed following earlier results of reduced capacity to xylose absorption. In all subjects, villous abnormalities observed, none could be classified as "normal" compared with apparently normal North Americans (Falaiye, 1971) suggesting the presence of EE in the Nigerians. EE, assessed using the L: M ratio, is reported to be widespread in rural Malawian children, where a group of investigators carried out several trials to try and ameliorate EE. Interventions tested include alteration of intestinal microbiome composition to improve small intestinal absorption capacity and the introduction of legumes to improve growth, rifaximin and probiotics (Trehan *et al*., 2015).

To date, in both children and adults, there are no robust biomarkers for EE. Lactulose (compared either to mannitol or rhamnose) is a commonly used biomarker, but investigators are actively looking for better biomarkers. Citrulline, a biomarker for gastrointestinal epithelial cell mass and function, was evaluated in one study in rural Malawian children aged 1-3 years. The results showed no correlation between and L: M ratio even in stunted children. Gouport *et al*. (2013) recruited non-stunted children with EE in Tanzania and obtained similar results (Gosselin *et al*., 2015) suggesting that cirulline is not a good biomarker of EE which is widespread in poor African settings especially in children. Yu *et al*. (2016) in Malawi performed a transcriptomic analysis on stool samples and found changes in transcripts related to cell adhesion molecules, reduced mucin expression, and immune activation. Further, in a different rural population in Malawi, it was demonstrated that the problem of EE has the potential to change the relevant metabolites that are involved in gut function and integrity also growth in children aged 12-59 months without

malnutrition. Metabolites such as tryptophan, ornithine, phosphatidylcholines, sphingomyelins, and citrulline were associated with increased gut permeability. Higher values of other metabolites such as taurine, serotonin, and glutamate in serum were also associated with increased gut permeability (Semba *et al*., 2016).

In Zimbabwe EE has been described as being prevalent among people living in areas of poor sanitation and hygiene especially children. The condition has been suggested to start early in life and perpetuate throughout life (Humphrey, 2009). A trial was conducted in Zimbabwe, under the Sanitation Hygiene Infant Nutrition Efficacy (SHINE) to test the hypothesis that EE is as a result of continuous exposure to faecal-oral contamination and undernutrition in children and is associated with stunting and other adverse effects. In this trial, children were protected from faecal-oral contamination and provision of adequate food and nutrients to determine if there would be improvements in health outcomes. Healthy practices and behaviours were promoted and responses to Water, Sanitation and Hygiene (WASH) interventions and some biomedical pathways measured intervention in infants could be explained by hormonal determinants of growth, systemic inflammation and microbial translocation. Some biomarkers that have been used are those that measure intestinal function and structure such as absorption, permeability, regeneration, and inflammation. To further understand whether EE interventions work or not, evaluation in the SHINE trial is underway (Prendergast *et al*., 2015) but a full report of the trial is not yet published.

Studies from animal models have shown that gut microbiota plays a significant role in growth and although this has not been shown in humans, the SHINE trial is trying to understand whether gut microbiota composition is linked to EE and child stunting (Gough *et al*., 2015). The changes in

microbiota are said to be associated with changes in innate and adaptive immunity (Gaboriau-Routhiau *et al*., 2009) which may influence EE. Furthermore, a study in Zimbabwe reported that EE is widespread and associated with stunting which begins during foetus development and progress in life (Prendergast *et al*., 2014).

2.3 Environmental Enteropathy in Zambia

In Zambia, a cohort study was conducted for 3 consecutive years in 200 adults above 18 years of age from an urban township with poor sanitation and hygiene where EE is reported to be ubiquitous. The study aimed to determine how different environmental conditions over a 3-year period would affect small intestinal mucosa architecture and function. Results from endoscopy and the intestinal biopsy, (Figure 2.2) revealed that virtually none of the participants had "normal" intestinal architecture. The study also reported that intestinal architecture varied with season and correlated with intestinal function (Kelly *et al*., 2004). The findings suggested that probably EE is widespread in Zambia. A follow-up study in the same population showed that the expression of both α-defensins HD5 and HD6 which are the major antimicrobial barrier in opposition to intestinal infection were reduced and appeared to change with season compared with adults living in London (Kelly *et al*., 2006). Results from these studies may suggest that since there are many areas with similar environmental conditions, EE may be widespread in Zambia with many adverse effects. The hypothesis that EE is as a result of environmental contamination and is widespread was further supported by the high prevalence and association with hepatitis E virus, which is also acquired from environmental contamination in the same adult population (Jacobs *et al*., 2013). More recently, the use of Confocal Laser Endomicroscopy (CLE) revealed that adults from an urban setting with poor sanitation and hygiene all had intestinal defects though the degree varied significantly and increased the problem of microbial translocation supporting the presence of EE

(Kelly *et al*., 2016). In another study, 81 volunteers recruited from among residents aged 18 to 60 years all had features of EE when endoscopic small bowel biopsies were performed (Louis-Auguste *et al*., 2014). The hypothesis that T-cell activation is critical in the pathogenesis of EE was tested by recruiting, 35 healthy black Zambian subjects without diarrhoea, malnutrition, or systemic illnesses were compared with 29 white South Africans. Duodenal biopsies were taken and quantified using a microscope after staining procedure. The investigators reported reduced villous height, increased crypt depth, increased mitoses per crypt, increased intraepithelial lymphocytes count and CD3+HLA-DR+, which is a marker of T-cell activation, in black Zambians compared with white South Africans (Veitch *et al*., 2001). All these findings are features of EE (Figure 2.2) and suggest the widespread prevalence of EE in the Zambian population.

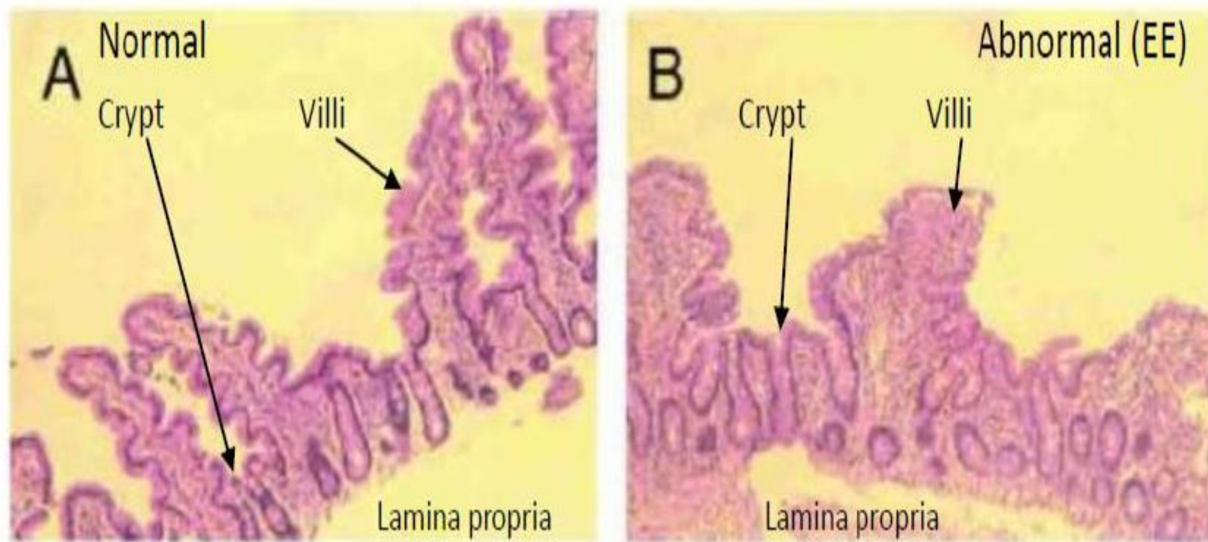


Figure 2.2: Shows normal intestinal mucosa (A) and (B) an abnormal mucosa (environmental enteropathy) (Source: Kelly et al., 2004).

2.4 What causes Environmental Enteropathy?

There is no known cause for EE, but it is hypothesized that continuous exposure to faecal-oral contamination leads to EE although there is no single organism that can be pointed out as the sole cause (Korpe and Petri, 2012). Many different microorganisms inhabit the human gut and play a significant role in growth and development especially in infants. The numbers are relatively few in the small intestine compared with ileum or colon. Individuals with poor bowel movement or inactivity and chronic exposure bacterial tend to develop small intestine bacterial overgrowth which is defined as bacteria count of more than 10^5 CFU/ml in the small intestine (Donowitz *et al*., 2016). In two separate studies in Brazil, researchers found similar rates of SIBO in children living in areas of poor sanitation and hygiene. One significant finding was that children who accessed private healthcare had less small intestinal bacterial overgrowth (SIBO) (dos Reis *et al*., 2007; Mello *et al*., 2012) suggesting that probably socioeconomic status plays a role in the development of EE. A study from Bangladesh reported that being exposed to faecal-oral contamination over time lead to changes in the types of bacteria in the small intestine. The more diverse the bacteria population, the more it was associated with malnutrition and growth faltering (Subramanian *et al*., 2014) suggesting EE.

2.5 Consequences of Environmental Enteropathy

2.5.1 Nutritional Deficiency

The World Health Organization recommends zinc as part of standard therapy for diarrhea (WHO, 2011). Zinc has been proposed together with other low osmolarity oral rehydration solutions (ORS), to reduce the duration and severity of diarrheal episodes and it is known to help in the recovery of the intestinal mucosa (Bajait and Thawani, 2011). In a Malawian study, children with EE were found to be zinc deficient and associated with abnormal L: M ratio which is a measure of

mucosal function (Ryan *et al*., 2014). In The Gambia, a study was conducted in children during the mango season when vitamin A intake is highest, and the results coincided with the observed least impairment in the gut integrity suggesting that vitamin A leads to improvement of gut integrity (Thurnham *et al*., 2000). These results were supported when similar results were reported in India by the same investigators when they compared children who were supplemented with vitamin A and those in the placebo group. In stunted Brazilian children with EE, it was reported that abnormal L:M ratio was associated with vitamin A deficiency (Chen *et al*., 2003) suggesting that probably vitamin A deficiency lead to reduced gut integrity. Attempts have been tried to treat EE by numerous nutritional approaches, including enzyme supplementation, improving the digestibility of food through fermentation and reducing gut inflammation while supporting repair through optimizing amino acid profiles (McKay *et al*., 2010). All these efforts have yielded less desirable results suggesting EE could be the probable cause. A dietary essential fatty acid, n-3 (omega-3) long-chain polyunsaturated fatty acid (LC-PUFA), which is believed to reduce intestinal inflammation, has been tried in The Gambia in children with EE, as an essential precursor for rapidly replicating cells like the ones lining the small intestine but the results were not satisfactory. However, alanyl-glutamine has similar mechanism of action and has shown some positive results in Brazil (Lima *et al*., 2010; Van der Merwe *et al*., 2013). These studies could add some insights into the nutritional causes of EE. There are very few dietary interventions that have been done in adults with EE.

In Zambian adults with environmental enteropathy without HIV infection were supplemented with high doses of multiple micronutrients which led to improved small intestinal villous height and absorptive area but not crypt depth (Louis-Auguste *et al*., 2014). Many vitamin A and zinc

nutritional trials among children in low- and middle-income countries have reported contradictory results on the EE biomarkers and growth (Lima *et al*., 2010; Radhakrishna *et al*., 2013; Ryan *et al*., 2014). Nutritional deficiency may contribute to the development of EE, but so far the dietary interventions have yielded minimal satisfactory results.

2.5.2 Reduced Oral Vaccine Response

Vaccines are substances that lead to stimulating the body to produce antibodies and provide immune protection against diseases and save many lives, especially in children. They have been highly regarded as cost effective with high impact, health measures and among the most affordable way of intervention to reduce diseases in populations especially in developing countries with high burden of diseases and were efficacious vaccines are most needed (Levine, 2010). However, in developing countries where EE is common the efficacies of many oral vaccines against enteric pathogens for instance poliovirus and rotavirus have been reported to have reduced effectiveness and this may have significant public health implications (Naylor *et al*., 2015). There may be several reasons for reduced response, but a few have been suggested. First, the reduction may be due to dampened vaccine-specific response by induction of regulatory T-cell leading to failure of immune system to mount an effective immune response during infection. Second, sometimes the presence of over-vigorous immune system in the gut due to the presence of many organisms may also destroy live attenuated vaccine (Prendergast and Kelly, 2012). Third, the other suggested mechanism for low efficacy is high maternal titers antibodies in breast milk that interfere with the oral vaccines (Chilengi *et al*., 2016). It is also possible for immunological disturbance to take place in the gut and both EE and reduced oral vaccine response may just be ultimate effects (Prendergast and Kelly, 2012).

Some investigators have reported reduced oral vaccines efficacy in developing countries, especially in sub-Saharan countries. The vaccine against rotavirus has been reported to have only 39.3% efficacy (Armah *et al*., 2010), while in Asia 48.3% (Zaman *et al*., 2010). The developing countries compare quite poorly with the efficacy of above 85% to almost 100% reported for Europe and USA combined (Vesikari *et al*., 2006, Ruiz-Palacios *et al*., 2006). In a cohort study in Bangladesh, infants were followed-up from birth to 1 year and were given all Expanded Programme on Immunisation (EPI). A reduced response to oral rotavirus of 80% of participants in the study with EE was reported. In the same study reduced oral vaccine response was also found to be associated with EE as well as biomarkers of systemic inflammation (Naylor *et al*., 2015). In children, poor oral polio vaccine (OPV) response has also been linked to undernutrition, EE, and stunting. In a cohort study in Bangladesh, stunted children had lower vaccine response compared with non-stunted children by 6 months of age. Additionally, the study suggested that both undernutrition and EE are associated with reduced OPV response due to the inverse correlation between serum EndoCab levels and OPV type 3 (Ogra *et al*., 2011). The trivalent OPVs have been reported in other countries with reduced efficacy of about 21% in India and many developing countries (Grassly *et al*., 2010) compared with 50% in the United States of America (Grassly *et al*., 2006). The reduced oral vaccine is common in areas where EE exists, and given this, there is a need to identify or improve diagnostic biomarkers of EE so that it can be managed. The suggestion is that this would also enhance the efficacy of oral vaccine particularly in settings where oral vaccines are most needed.

2.5.3 Growth Faltering and Malnutrition

The small intestine has two primary functions; not only does it absorb nutrients but it also excludes bacteria and other food antigens from entering the systemic circulation. All these can be altered in

malnutrition resulting in growth faltering. More than 10 years, studies have attempted to promote exclusive breast feeding and provision of adequate supplementary foods have also been made to try and solve the problem of growth faltering in children especially in developing countries. These efforts have achieved minimum results (Asfaw, 2007; Yisak *et al*., 2015; Fekadu *et al*., 2015). Studies have reported that there is a huge gap to tackle the problem of malnutrition in developing countries. It has been demonstrated by a study that even with the best available interventions, 90% of children with stunted linear growth from 36 countries; mortality has been reduced by only a quarter and stunting close to one- third (Bhutta *et al*., 2008). To solve the problem of malnutrition and growth faltering, many interventions that have been used have produced low beneficial outcomes party due to EE (Subramanian *et al*., 2014).

Developing countries have an enormous burden of growth faltering (height-for-age Z score < -2) with an estimated 32% of children under the age of 5 years while 20% of children are malnourished (weight-for-age Z score < -2). One of the suggested risk factors of growth faltering is the increased intestinal pathogens early in life (Black *et al*., 2008). Growth faltering has been previously shown to correlate with increased gut permeability in children with the age range of 2 to 15 months (Lunn *et al*., 1991), probably suggesting that breast feeding and adequate food cannot solve the problem of growth faltering. The suggestions have been that the integrity of the gut damaged by EE needs to be considered especially for children in poor communities. Irreversible growth faltering happens within the first 2 years of life (Black *et al*., 2013) and that is why it is imperative to put necessary measures in place such as to avoid EE to achieve optimal growth. Increased gut permeability and altered intestinal morphology usually go together with malnutrition and compounded with chronic inflammatory responses in growth faltering. EE is known to reduce absorption of micronutrients

(Manary *et al*., 2010) so the integrity of the gut mucosa and nutritional status are strongly linked (Manary *et al*., 2010; Lindenmayer *et al*., 2014). However, there are few studies of the impact of EE on micronutrient absorption. The Malawi group, using stable isotopes, reported that in children who were taking a diet low in animal protein but high in carbohydrate diet, the amount of zinc excreted in stool was higher than expected. They suggested that the endogenous reabsorption of zinc was negatively affected by EE (Manary *et al*., 2002). Another study followed-up by selecting children who had higher L: M ratio and a positive association with the amount of zinc excreted in stool was found led to the conclusion that there is failure of zinc reabsorption in EE probably due to loss of intestinal integrity. Other conditions with similar histological pictures like EE such as celiac disease have reported both increased fecal zinc excretion (Crofton *et al*., 1990) and reduced gut function as well as absorption of zinc (Tran *et al*., 2011).

Once EE is established, it may reduce the absorption of zinc. Intestinal barrier function may also be impaired due to zinc deficiency, so this is believed to be a bi-directional process constituting a vicious cycle in which zinc malabsorption and the mucosal effects of zinc deficiency worsen each other. In EE there is intestinal inflammation, increased permeability, malabsorption, microbial translocation, chronic immune activation and together these eventually result in growth faltering (Lindenmayer *et al*., 2014). In putting up public health measures to tackle the problem of EE or zinc deficiency, both should be considered together since interplay seems to exist. A scheme of interaction among several factors that may affect growth, stunting due to EE is shown (Figure 2.3).

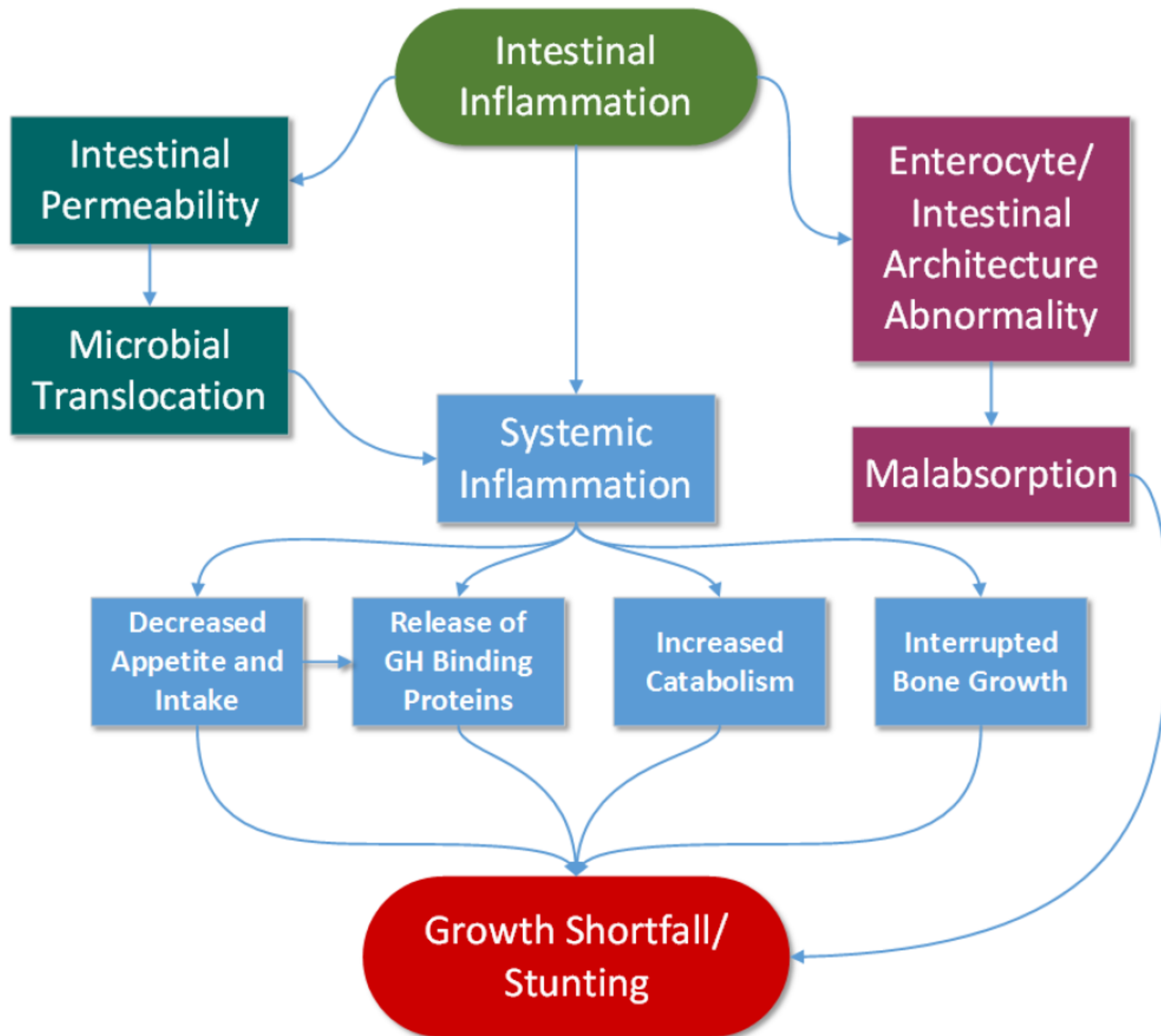


Figure 2.3: Possible pathophysiological processes that are EE related that result in stunting/growth faltering (Source: Denno et al., 2016).

2.5.4 Poor Neurocognitive Development

In animal models, studies have reported that inflammatory processes before birth cause poor neurocognitive development in the off spring later in life (Dinel *et al*., 2014; Bland *et al*., 2010; Spencer *et al*., 2005). Inflammation due to LPS exposure has also been suggested as a cause for poor neurocognitive development (André *et al*., 2014), however, in humans, it has not been shown. But studies have reported that diarrheal episodes, some parasitic infections such as

Cryptosporidium spp. (Guerrant *et al*., 1999), *Giardia intestinalis* (Berkman *et al*., 2002) and stunting in children are linked to poor neurocognitive development in later life (Mendez and Adair, 1999; Guerrant *et al*., 1999; Berkman *et al*., 2002). One of the essential minerals is iron which has several important roles in the body, including development and function of the brain (Yehuda and Mostofsky, 2010). Iron deficiency leads to behavioural and cognitive developmental delays, but the mechanism of how it happens is still under unclear. Some mechanisms have been put forward to explain the role of iron in brain development. First, there are certain enzymes such as histone deacetylase 2 (HDAC2), in the brain that contain iron and are responsible for fatty acid concentration in the myelin and under conditions of iron deficiency, there is a decrease in the myelin function leading to a neuropathology (Feliciano and Bordey, 2013). Second, alteration of the brain energy metabolism due to unproductive ATP generation and pasting of cytochrome as well as reduced electron transfer and also, abnormality in neurotransmitter metabolism.

Unfortunately, some studies have reported that effects of iron deficiency in children are irreversible (Radlowski and Johnson, 2013) although mainly depends on the timing of the deficiency which has been proposed to be between 3 – 16 months. In humans, the conclusion has been based on the number of intervention trials in attempts made to correct the deficiency. However, utilization of animal models where iron-deficiency is induced has significantly helped to understand timed models (Siddappa *et al*., 2002). Some recent studies have also reported that early or late iron-deficiency affects the expression of genes involved in some processes including cellular energetic in the brain and upregulation of certain pathways (Carlson *et al.,* 2007). One such pathway is upregulation of the mechanistic target of rapamycin (mTOR) pathway, an intracellular signaling that senses cellular nutrient and energy levels. Due to the effect iron levels

have on mTOR pathway, deficiencies in the body may result in dysfunction of the brain (Kaplan *et al*., 2014). In environments where EE, stunting and parasitic infection are common, the absorption of iron can be hampered leading to one possible mechanism of how the iron deficiency can bring about poor cognitive neurodevelopment. Therefore, it is probably important when poor cognitive neurodevelopment in children is being mitigated to consider all these factors together.

2.5.5 Microbial Translocation

There are several other consequences of EE but of importance to this study is microbial translocation. Microbial translocation is the passive movement of microbes and/or their products across the intestinal barrier into the mesenteric lymph nodes, blood circulation and other sterile sites (Marchetti *et al*., 2013). The human intestinal epithelial layer is one cell thick and has two major roles, absorption of food and other nutritional requirements for the host and exclusion of bacteria and other food antigens from entering sterile sites such as mesenteric lymph nodes and the blood. The human gut has many species of bacteria, mostly non-pathogenic, which have a symbiotic relationship and are part of its normal microbiota. In good health, the human gut contains around 10^5 colony forming units (CFU)/ml of non-pathogenic bacteria in the jejunum, around 10^8 CFU/ml in the distal ileum and cecum and up to 10^{12} CFU/ml in the colon (Marteau *et al*., 2001). Dominant taxonomic groups include Actinobacteria and Proteobacteria, Firmicutes and Bacteroidetes (Arumugam *et al*., 2011). About 1,000 microbial species belong to Firmicutes, or Bacteroidetes species cannot be cultured and this makes identification of these organisms by usual laboratory culture techniques sophisticated (Brenchley and Douek, 2012). Currently, the link between human gut and microbiota has not been completely understood. Furthermore, not a lot of work has been done to understand the immune response to gut microbiota in humans fully.

2.6 Hepatosplenic Schistosomiasis

Hepatosplenic schistosomiasis (HSS) refers to the main complication of chronic infection caused by a number of Schistosoma species including but not limited to *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi* and *Schistosoma intercalatum*. HSS denotes a distinct mode of intrahepatic portal hypertension characterised by a spared liver parenchyma and a partly blocked portal vein. (Sharker *et al*., 2014; Marinho 2010)

2.6.1 Epidemiology of Hepatosplenic Schistosomiasis

Globally, approximately more than 200 million people are infected with schistosomal species and about half of those infections are due to species that cause HSS infection. The highest burden of the disease is in Africa with about 60% of population live in areas with the possibility of being infected and up to about 40% of them are infected (Esmat *et al*., 2000; Chitsulo *et al*., 2014). Some studies have suggested that *S. intercalatum,* as well as *S. mansoni*, are common in the west and central Africa but *S. mansoni* dominates most parts of central Africa and chronic heavy infection may lead to hepatosplenomegaly (Gazzinelli *et al*., 2012). However, in Africa generally, *S. mansoni* species is by far the common cause of HSS infection. Although the distribution of *S.mansoni* vary greatly in Africa, it is less in some countries such as the Congo Republic (Brazzaville), Somalia, Algeria, Mauritania, Niger Tunisia and Morocco but in the same countries some regions may have high prevalence (De cook et al., 1983; Abebe *et al*., 2014). In Kenya, when some HSS patients were assessed, there was evidence of oesophageal and gastric varices in about 33% who had portal hypertension (Wallen, 1983). In some studies, factors such low platelet count, enlarged spleen size, increased portal diameter, increased varices are some of the major predictors of oesophageal bleeding and later portal hypertension (Mazigo *et al*., 2015). Generally, the world

over cirrhosis in which the liver parenchyma is not spared is the major cause of portal hypertension but in the tropical countries, it is HSS infection which is the leading cause (Shaker *et al*., 2014).

The pathophysiology of HSS infection is related to the intensity of eggs and the worms themselves as they increase, the immune system responds leading to the formation of granulomata resulting into chronic fibrosis with little or no resolution, subsequently may lead to the development of portal hypertension. Furthermore, some studies have reported some fascinating findings that extra collagen could be deposited independently of granulomata worsening the HSS disease. A further interesting observation is that excess collagen may be deposited independently of any granuloma in the spaces of the sinusoids resulting in more fibrosis and genetic factors have been suggested as partly contributing to the development of HSS disease in certain individuals (Herbert *et al*., 2008). With similar levels of infection, HSS has been reported to occur more in the white than black people and being more common in the blood group A individuals (Camus *et al*., 2007).

In Zambia, the prevalence of *S. mansoni* in specific areas is as high as 77% (Chipeta *et al*., 2009) while others have reported sero-prevalence of about 88% in the western province of Zambia (Payne *et al*., 2013). Moreover, on stool examination using Kato-Katz method, another study found 42% prevalence of *S. mansoni* in the same area (Mutengo *et al*., 2014). More recently another study reported higher surrogate biomarkers of microbial translocation in patients with HSS disease compared to healthy controls (Sinkala *et al*., 2016) suggesting that there was more microbial translocation in the HSS patients compared to healthy controls.

Some studies have reported that overgrowth of microorganisms in the small intestine may contributes to portal hypertension if organisms move into systemic circulation. With the increased

pressure from portal hypertension, gastric and oesophageal varices may bleed and may be the source of microbial translocation. Microbial translocation is the movement of microbes' from the gastrointestinal tract across intestinal mucosa into the stile sites such as systemic circulation. Factors such as increased intestinal permeability and microbial overgrowth in the intestines contribute to increased microbial translocation and is one of the main source of morbidity and mortality in HSS disease (Bellot *et al*., 2013. Direct biomarkers that can be used in HSS disease to measure microbial translocation include lipopolysaccharide, 16S rRNA gene and detection of Toll-like receptor ligands using RAW-Blue cell line that can indicate the total PAMPs activity present in blood. Uncontrolled microbial translocation can lead to chronic immune activation of the innate system and uncontrolled immune activation may result in microcirculatory dysfunction, septic shock, morbidity and mortality (Mocellin *et al*., 2004). The main driver of immune response are the cytokines such as Tumour Necrosis Factor-alpha (TNF α), Interleukin 4 (IL-4), Interferon gamma (IFN-γ), Interleukin 6 (IL-6) and Interleukin-4 (Yu *et al*., 2012).

2.6.2 Diagnosis of Hepatosplenic Schistosomiasis

The gold standard in the diagnosis of schistosomiasis is finding eggs in stool or rectal biopsy (Smith *et al*., 2012) but others have suggested that in advanced HSS disease, finding eggs in stool examination is uncommon and therefore, it is important to consider antibody detection against parasite antigens using ELISA with good sensitivity and specificity which can detect the infection in both acute and chronic stages (Ross *et al*., 2002). Other diagnosis methods are ultrasonography which can help in the quantification of HSS disease (Silva *et al*., 2011).

2.7 Exclusion of Microorganisms by Mucosal Barrier

The primary composition of the intestinal mucosa includes the muscularis mucosa, lamina propria and epithelium (van der Flier *et al*., 2009). Immune cells, mostly the ones belonging to the innate immune system such as dendritic cells, mast cells and macrophages, together with the lymphoid system, monitor the intestinal mucosa. Under healthy conditions, the intestinal mucosa is protected by a gel-formed barrier, composed of mucins secreted by goblet cells. The compositions form a protective barrier for the mucosal layer to have direct contact with bacteria and other antigens. The mucosal layer components consist of water, electrolytes, phospholipids, and proteins (Bry *et al*., 1994). Besides, there are antimicrobial agents such as the defensins HD5 and HD6, as well as IgA, which have the ability to hinder bacteria from crossing the intestinal barrier (Duerr and Hornef, 2012) together with many other cells and biological molecules. These include enterocytes involved in absorption and hormone production. They have a short lifespan and are regularly replaced within a few days. Also, other cells such as paneth cells are also found in the crypts and are involved in the production of growth hormones, digestive enzymes and defensins (Bry *et al*., 1994). Furthermore, there is a gastric barrier acid which kills many ingested bacteria and other pathogens. Other components of the intestinal mucosa are villi and crypt, which are significant in the absorption of nutrients, secretion of fluids containing electrolytes and serve some immune functions (Turner, 2009; Peterson and Artis, 2014). The villi are finger-like protrusions that increase the surface area for absorption and the crypt are at the bottom of villi and secrete essential fluids containing antimicrobial peptides. Altogether these different elements form an intestinal mucosa barrier with immune and absorptive functions (Mbuya and Humphrey, 2015).

The enterocytes are joined to each other or to adjacent cells by a complex of tight junction proteins which is composed of claudins, ZO-1 and occludin, and play a significant role in the selective regulation of ionic solutes passing between the cells. In case the intestinal barrier is compromised,

resulting in marked loss of barrier function, microbes or their products cross the barrier through the paracellular pathway (Turner, 2000). The paracellular pathway is more permeable than transcellular pathway, which involves passive passage of substances through the cells themselves. Both the transcellular and paracellular pathways involve the action of specific channels, which move materials passively or actively across cell membranes (Turner, 2009). Evidence suggests that these two routes are possibly controlled autonomously (Fihn *et al*., 2000; Watson *et al*., 2005). Tight junction proteins include the family of 18 claudins, which play a significant role in the determination of pole charge. Other proteins which play important roles in the epithelial barrier function include desmosomes and adherens which form junctions joined to the actin cytoskeleton (Bruewer *et al*., 2005; Turner, 2009). Many *in-vivo* and *in-vitro* studies have demonstrated that lipopolysaccharide and TNF-α increase the permeability of the gut, thereby, exacerbating microbial translocation (Watson *et al*., 2005; Clayburgh *et al*., 2005). However, it is not known whether transcellular or paracellular pathways are responsible for the greater part of microbial translocation, or in which disorders they predominate. In the presence of ulceration, paracellular translocation predominates, but when there is no visible defect in epithelium it is not clear which is quantitatively more important.

2.8 Assessment of Intestinal Barrier Integrity

Intestinal barrier integrity can be assessed by staining the intestinal biopsy with Hematoxylin and Eosin (H and E) and reveal any alterations of the mucosa (Kelly *et al*., 2016). The technique is of particular importance in demonstration of the composition and integrity of tight junction proteins occludins and claudins, as well as zonula occludens (Menard *et al*., 2010). These proteins can also be studied in detail by immunofluorescence staining using antibodies that bind specifically to proteins under investigation (Hartmann *et al*., 2012). Some tight junction proteins such as claudin

4 have also been evaluated using Western blotting (Kelly *et al*., 2016). There are many more proteins involved in maintaining the integrity of the tight junctions and because of the complexity of their interactions *in-vivo* it may not be possible to single out the single most important protein involved in the maintenance of barrier integrity. One of the most promising developments in understanding the histology of the mucosa *in-vivo* is the use of confocal laser endomicroscopy, which is capable of revealing small gaps within the epithelial layer and has already been used in studying environmental enteropathy (Kelly *et al*., 2015; Kelly *et al*., 2016).

2.9 Clinical Significance of Microbial Translocation

There is substantial literature on experimental studies that have demonstrated that microbial translocation occurs in animal models under various conditions. Factors that have been reported to be associated with microbial translocation are diverse. These include EE (Brown *et al*., 2015), colitis (Hao *et al*., 2015), liver cirrhosis (Wiest *et al*., 2014) and small bowel obstruction. Others are ischemia in vivo (Samel *et al*., 2002), haemorrhagic shock (Fülöp *et al*., 2013), and trauma (Zanoni *et al*., 2009). Abuse of opiates such as morphine (Meng *et al*., 2013) and acute pancreatitis (van Minnen *et al*., 2007) has also been reported to cause microbial translocation. However, in humans, the clinical significance of microbial translocation remains to be elucidated, and some conditions where some studies have been done are discussed in the sections that follow.

2.9.1 Microbial Translocation in Environmental Enteropathy

It has been suggested that people living in areas of poor sanitation and hygiene are affected by a widespread phenomenon of asymptomatic abnormal structural and functional changes of the small intestine referred to as environmental enteropathy (Korpe and Petri, 2012). EE has no known cause but it has been hypothesized that repeated exposure to faecal-oral contamination may play a

significant role in the development of environmental enteropathy. The condition is associated with some problems, including reduced responses to oral vaccines (Levine, 2010; Naylor *et al*., 2015) and micronutrient deficiencies. Others are growth failure, stunting in children (Prendergast *et al*., 2014) and microbial translocation (Campbell *et al*., 2003; Kelly *et al*., 2016). It is a subclinical condition and characterized by loss of intestinal barrier function, chronic intestinal inflammation, microbial translocation and chronic immune activation (Korpe and Petri, 2012; Prendergast and Kelly, 2012; Keusch *et al*., 2014). Due to compromised gut barrier, microbes and/or their products translocate from the gut into systemic circulation resulting in immune activation. Chronic immune activation may lead to microcirculatory dysfunction, intravascular coagulation and hemodynamic disturbances leading to hypotension, metabolic derangements, septic shock and death (Zanoni *et al*., 2009).

2.9.2 Microbial Translocation in Hepatosplenic Schistosomiasis

Chronic infection with *Schistosoma* species, including *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma intercalatum* and *Schistosoma mekongi*, may lead to hepatosplenic schistosomiasis (HSS). This results in portal hypertension leading to the formation of gastric and/or oesophageal varices as shown in Figure 2.4 (Sinkala *et al*., 2016). Increased venous pressure may contribute to the development of varices which can bleed resulting in anaemia, severe haemorrhage and death. Few studies have reported biomarkers of microbial translocation in HSS patients, but a recent study in Zambia showed that surrogate biomarkers of microbial translocation such as lipopolysaccharide-binding protein (LBP), soluble CD14 and CD163 were elevated in HSS patients compared with healthy controls suggesting that microbial translocation occurs in HSS patients (Sinkala *et al*., 2016). A similar study in Brazil reported that in patients with HSS disease with portal hypertension, microbial translocation may contribute to postoperative infection

complication that may arise (Ferraz *et al*., 2005). In western Kenya, it was observed that patients with *Schistosoma mansoni* infection had elevated levels of EndoCab, suggesting microbial translocation (Onguru *et al*., 2011). The gastric varices that are formed in HSS patients are similar what is shown in Figure 2.4.

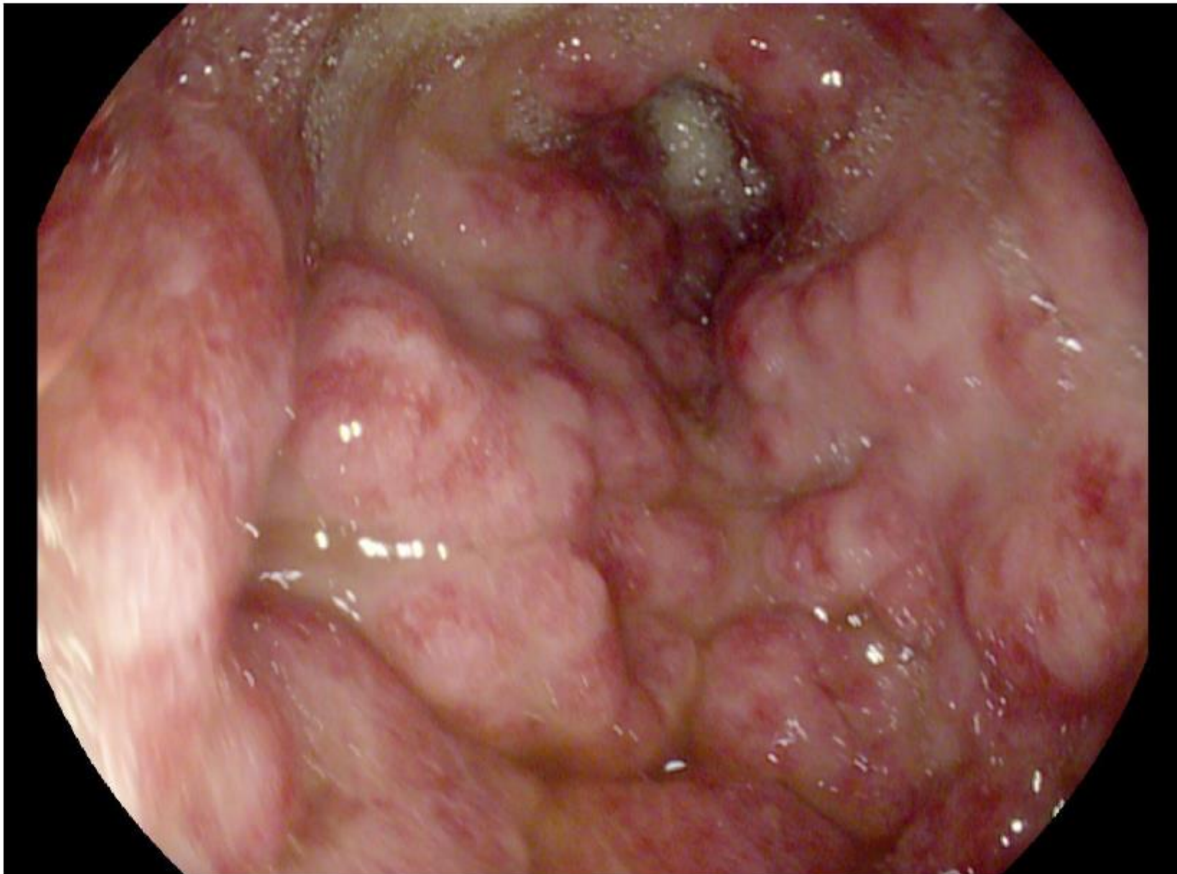


Figure 2.4: A picture showing gastric varices in an adult Zambian patient with hepatosplenic schistosomiasis (Source: Sinkala et al., 2014).

2.9.3 Microbial Translocation in HIV Infection

HIV is known to infect the lymphocytes and macrophages of the intestinal mucosa (Brenchley and Douek, 2012). In severely immunosuppressed humans living with HIV and particularly those with full-blown AIDS, chronic diarrhoea and weight loss are common. In such individuals, the gut

appearance at the microscopic level is similar to that of EE (Kelly *et al*, 2004). HIV damages the mucosal immune system, allowing pathogens to damage the epithelium culminating into enteropathy (Crane *et al*., 2015). Some *in vitro* experiments have reported that the HIV glycoprotein gp120 interrupts the tight junction proteins (Nazli *et al*., 2010. The suggested effects on the gut mucosa in HIV patients including paracellular permeability has been demonstrated by using immunohistochemistry techniques (Buccigrossi *et al*., 2011). Taken together, these increase the chances of microorganisms in the gut to move across the gut barrier. On the contrary, a study in EE individuals showed no difference in L: M ratio, a marker of permeability when HIV sero-positive individuals were compared with HIV sero-negative (Kelly *et al*., 2016) suggesting that maybe EE masked the effect of HIV.

2.9.4 Microbial Translocation in Patients with Liver Cirrhosis

Microbial translocation has been reported to occur in about 30% of patients with cirrhosis (Cirera *et al*., 2001) and has been demonstrated to be even as high as 78% in a mouse model of cirrhosis. A study showed that 16S rRNA, a direct biomarker of microbial translocation, was elevated in patients with cirrhosis (Bellot *et al*., 2013). The 16S rRNA has been suggested as a biomarker of microbial translocation by others as well but with no correlation with the severity of cirrhosis. Another study demonstrated that cirrhotic patients had elevated LBP compared with healthy control group and concluded that there was a possible involvement of bacteria and/or their products. The condition was improved by antibiotics (Albillos *et al*., 2003). Others have demonstrated microbial translocation from the positive bacteriological culture from surgical removal of mesenteric lymph nodes (MLN) in animal models (Pinzone *et al*., 2012). In a study of cirrhotic patients who were treated with rifaximin, an antibiotic which acts local in the gut reported

reduced portal pressure (Vlachogiannakos *et al*., 2009) suggesting that microbial translocation was reduced.

The pathogenesis of microbial translocation in cirrhosis may manifest in different ways, including small intestinal bacterial overgrowth (SIBO) which most investigators define as 10^5 CFU/ml of proximal jejunal aspirate (Sachdev and Pimentel, 2013). However, the primary challenge for diagnosis of SIBO procedure using proximal jejunal aspirate is the invasiveness of the procedure. For this reason, the non-invasive method of the hydrogen and methane breath tests after an oral dose of glucose or lactulose is preferred (Vanderhoof and Pauley-Hunter, 2016). Microbial translocation was only present in about 50% of cirrhotic with SIBO mice suggesting that other factors apart from SIBO were responsible for microbial translocation in mice. Some investigators have reported structural and functional alterations in cirrhosis which predisposes mice to microbial translocation. The general immunological impairment of the intestinal immune system has been shown to promote microbial translocation in cirrhosis (Bellot *et al*., 2013). Taken together, these results may partly explain why microbial translocation in patients with cirrhosis is common.

2.9.5 Microbial Translocation in Inflammatory Bowel Diseases

The exact cause of inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC) is still elusive. The most favoured hypothesis currently is that genetically predisposed individuals develop an abnormal innate and adaptive immune response to the intestinal microbiota (Sartor, 2014). Studies have shown that patients with CD and UC have higher levels of plasma and tissue bacterial DNA compared with healthy controls suggesting microbial translocation (Pastor Rojo *et al*., 2007). The levels of bacterial DNA are also higher in patients with active disease than in those with the inactive disease. Other investigators have reported that

bacterial DNA levels are not associated with microbial translocation (Vrakas *et al*., 2017). On the contrary, different studies showed that bacterial DNA in the serum of IBD patients was is associated with microbial translocation. The results were further supported by elevated levels of pro-inflammatory cytokines (Gutiérrez *et al*., 2009; Gutiérrez *et al*., 2011). Some studies have demonstrated that *Bacteroides* species are more commonly found in intestinal biopsies of CD patients compared with those with irritable bowel syndrome or healthy controls. The studies have noted that significant increase in both CD and UC whether active or inactive compared with healthy controls may be linked to some factors including smoking, diet, and biopsy or stool sample. Variations in the activity of the disease may be explained by other factors including sample size (Bililoni *et al*., 2006; Swidsinski *et al*., 2005).

2.9.6 Other Conditions in which Microbial Translocation is of Clinical Significance

In industrialized countries, one of the causes of liver disease is a non-alcoholic fatty liver disease (NAFLD). Mostly happens when there are insulin resistance and obesity compounded with steatohepatitis which may originate from simple steatosis. Some investigators have suggested that obesity is associated with alteration in the gut microbiota (Tilg *et al*., 2009) and increased intestinal permeability which may lead to microbial translocation (Brun *et al*., 2007; Miele *et al*., 2009). In one study, 96 neonates and infants who required parenteral nutrition due to gastrointestinal abnormalities successfully underwent surgical procedures. They were followed up for the development of septicaemia due to microbial translocation in association with parenteral nutrition. Blood samples were cultured to diagnose microbial translocation and samples from 15 patients were found to be positive for bacteria with almost half of them associated with sepsis (Pierro *et al*., 1996). In 50 paediatric patients, who were immunosuppressed and were about to undergo small bowel transplant, the correlation between microbial translocation and acute rejection or

preservation injury was evaluated. A positive culture from blood or liver biopsy was considered evidence of microbial translocation. In some cases, microbial translocation was associated with colon allograft, ischemia and acute rejection (Cicalese *et al*., 2000). In monitoring postoperative sepsis in patients after undergoing laparotomy, patients were divided into those who had a positive bacterial culture in mesenteric lymph nodes and those who were negative. After a comparison had been made, patients who had a positive culture (microbial translocation) had more sepsis compared with those with negative culture (42.3% versus 19.9%) respectively (MacFie *et al*., 2006). In another study, at the time patients were undergoing general surgery, intestinal serosa and MLN samples were taken. After culture, only 10% had microbial translocation with the occurrence of postoperative sepsis being two times more than in patients with microbial translocation but with a similar mortality rate (Sedman *et al*., 1994).

Investigators have proposed two possible pathways of microbial translocation, one through the lymphatics and MLNs, and the other directly into portal blood (MacFie *et al*., 2006). The diagnosis of microbial translocation by positive culture of a sample taken from MLNs is regarded as the 'gold standard' measure of microbial translocation by most investigators but it has several limitations. First, to get such a sample, surgery is needed. The procedure is very invasive and would be difficult to justify in humans. Second, not all bacteria are cultivatable and so negative growth may not mean that MLNs are free of bacteria. Third, culture is not very sensitive because a certain quantity of bacteria needs to be present to get a positive result compared with methods such as PCR. Fourth, even if samples of MLNs were to be obtained, it is not practical to get samples from all MLNs because if certain MLNs are found to be negative, it may not necessarily mean all MLNs do have bacteria. Fifth, some bacteria may be present in the non-viable state and so may not grow on culture media (Oliver, 2005).

2.10 Is there a Specific Biomarker of Microbial Translocation?

After microbes and/or their components cross the intestinal barrier, the innate immune system composed of unspecialized cells detect all antigens from pathogen referred to as Pathogen-Associated Molecular Patterns (PAMPs). PAMPs are components of microorganisms which stimulate the immune system. The detection is through Pathogen Recognition Receptor (PRR) such as Toll-like receptors (TLRs). TLRs are germ-line encoded conserved receptors that are either expressed on the surface or endosomal that recognise various PAMPs (Akira *et al.,* 2006). In humans, there are nine TLRs that have been described and recognise different PAMPs. PRRs are an ancient system for recognising antigens. Only vertebrates have adaptive immunity, so for the vast majority of animals and plants, PRRs represent the only mechanism for avoiding or limiting pathogen spread.

Several methods have been proposed for the diagnosis of microbial translocation in humans with varying successes and limitations. These methods are based on various types of PAMPs, including lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan (PDG) components, flagellin, and their interactions with cell receptors. LPS is a component of the Gram-negative bacterial cell wall which has been detected in the plasma of human with EE (Campbell *et al*., 2003; Kelly *et al*., 2016) and hepatosplenic Schistosomiasis (Sinkala *et al*., 2016). Other conditions are in cirrhosis and HIV enteropathy (Cassol *et al*., 2010). Some investigators have considered LPS as a useful biomarker of microbial translocation because it has a short half-life (2 – 3 hours) typically affected by some factors such as antibodies, immunogenetic and physiological variables (Opal and Esmon, 2002).

In some conditions, detection of 16S rRNA, is a component of the 30S ribosome RNA in prokaryotic cell, in plasma of both human and animals has been used as a direct biomarker of microbial translocation (Marchetti *et al*., 2013).

2.11 Detection and Quantification of 16S rRNA genes in Blood by Quantitative Real-time Polymerase Chain Reaction

The 16S rRNA is a conserved studies have reported higher 16S rRNA copy number in chronic HIV infected individuals with lower CD4 count compared with HIV negative individuals (Jiang *et al*., 2009; Cassol *et al*., 2010; Kramski *et al*., 2011). Others found HIV infected treatment naïve individuals had higher copy number in blood compared with HIV infected persons who were on treatment (Brenchley *et al*., 2006). Others have also reported the presence of 16S rRNA in blood of healthy individuals (Moriyama *et al*., 2008; Ferri *et al*., 2010) probably confirming what others have reported that even in healthy persons with intact epithelial barrier translocation takes place.

3.12 Detection of Other Pathogen-Associated Molecular Patterns

The peptidoglycan layer is a component of Gram-positive and Gram-negative bacteria which is detected by Toll-like receptor 2 (TLR2) (Purohit *et al*., 2008). It comprises about two-thirds of the gram-positive cell wall and one-fifth of the gram-negative cell wall. It has been detected in human plasma using the silkworm larvae test. Although initial experiments were not done in humans, its later use in some patients during the postoperative period of gastrointestinal surgery (Kobayashi *et al*., 2000) revealed its potential for application in humans. Peptidoglycan levels were found to be higher in more than three-quarters of patients with severe bacterial infection (Shimizu *et al*., 2005). Conversely, its use as a universal biomarker of microbial translocation has been challenging.

Flagellin is a subunit component of flagella present in motile bacteria (Cole *et al*., 2010) and an ELISA assay has been developed capable of detecting the protein. One of its first uses was in patients with short bowel syndrome who had either endotoxemia or without and in these patients marked increase in serum IgM, IgA, and IgG levels specific to flagellin were observed (Ziegler *et al*., 2008). In patients with CD, other investigators have reported presence of flagellin unique to Escherichia coli (Sitaraman *et al*., 2005; Targan *et al*., 2005) which was associated with compromised gut barrier. In the treatment of patients with HIV infection, anti-flagellin antibodies were used as biomarker of microbial translocation (Vesterbacka *et al*., 2013). The use of this PAMP has been very limited, and this warrants more studies to investigate its viability as a biomarker of microbial translocation in different diseases.

Lipoteichoic acid (LTA) is another PAMP that has been proposed as a diagnostic biomarker of microbial translocation. It is an equivalent of LPS in the gram-negative bacteria, and it is shed during gram-positive bacteria replication. It has been shown to induce the production of cytokines that are different from the ones observed when LPS stimulate transcription factors. If bacteria are exposed to antibiotics in the culture medium, LTA is secreted, and in human, its titers have been shown to be higher in patients with chronic hepatitis C compared with healthy individuals (Haruta *et al*., 2006). Other studies have demonstrated that in patients with primary biliary cholangitis, LTA containing in plasma and in mononuclear cells were found in histological sections (Tsuneyama *et al*., 2001) suggesting microbial translocation.

2.13 Biomarkers of Host Response to Microbial Translocation

Indirect (surrogate) biomarkers are cellular biological molecules produced as a result of host immune response to microbial translocation. Several biomarkers are reported to correlate with

microbial translocation. These include soluble CD14, a core factor (alongside TLR4/MD2) in detection of LPS (Brenchley *et al*, 2006; Prendergast *et al*., 2014), lipopolysaccharide-binding protein (Sinkala *et al*., 2016) which enable the diffusion and transfer of bacterial LPS and C-reactive protein, a non-specific inflammatory biomarker produced by the liver (Pastor Rojo *et al.,* 2007). Others include CD163, a Kupffer cell activation biomarker (Pasternak *et al*., 2007) and EndoCab (Pelsers *et al*., 2003). However, other authors have argued that sCD14 is not a biomarker of microbial translocation *per se* but rather a biomarker of monocyte activation but correlates well with LPS (Anderson *et al*., 2010). Another potential measure of direct biomarkers of microbial translocation is the use of plasma which stimulates a reporter cell line such as RAW-Blue mouse macrophages to express TLRs capable of detecting total PAMPs readout which are of microbial origin (InvivoGen, 2010).

2.14 How Immune System Detects Microbes and Microbial Products

2.14.1 Toll-like Receptors

Toll-like receptors (TLRs) are a type of PRR that occur as membrane proteins expressed by almost all mammalian cells. They typically contain 10 domains comprised of leucine-rich repeats (LRRs) that are found on the cells of innate immune system such as macrophages and dendritic cells. These recognize structurally conserved molecules of microbes. When microbes have breached the physical barrier, they are recognized by TLRs (Janssens and Beyaert, 2003). TLRs 3, 7, 8 and 9 are located on endosomes, which detect PAMPs of nucleic acid origin and usually signify intracellular viral replication (Janssens and Beyaert, 2003) and signaling pathways are initiated so that the immune system can destroy the microbes. If the immune response is excessively prolonged and intense, the effects can be detrimental, and it is the TLRs that alert the host about the PAMPs. In humans, there are nine TLRs (TLR1 to TLR9) (Kanzler *et al*., 2007). TLRs 1, 2, 4, 5 and 6 are

located at the cell surface and are responsible for the detection of PAMPs of microbial outer membrane origin (Janssens and Beyaert, 2003) as illustrated below (Figure 2.5).

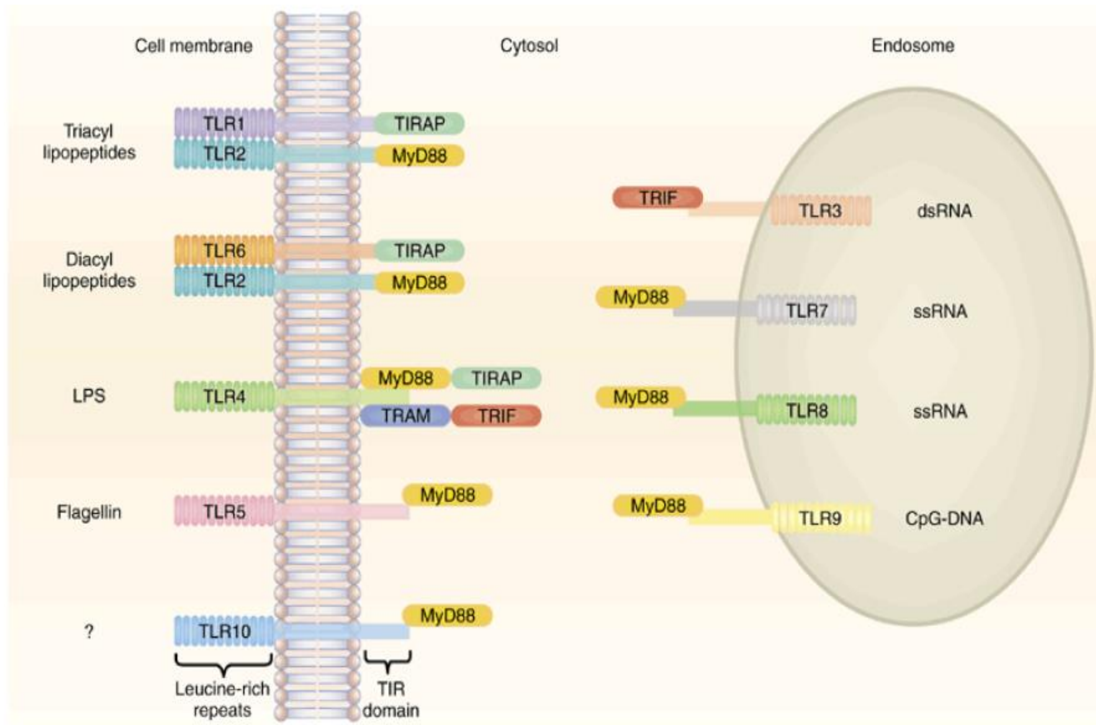


Figure 2.5: Toll-like receptors 1-9 and their interaction proteins in humans (Source: Kanzler, 2007).

TLRs recognise PAMPs including flagellin, teichoic acid, and lipopolysaccharide which are conserved and structurally integral to microorganisms' survival (Medzhitov *et al*., 1997). TLRs can recognise and interact with PAMPs as non-self-molecules as described above. Firstly, PAMPs are essential for microbial survival and indispensable for life as such loss of patterns, or even mutation can mean the loss of life, so they have low significant mutation rates. Secondly, they are not produced by host cells. Instead, they are produced by microorganisms, and this allows innate immunity to distinguish between non-self and self. Thirdly, between organisms of a given class,

PAMPs are invariant, meaning that not a lot of encoded PRRs are needed for microbial infection presence to be detected (Medzhitov and Janeway, 1997).

2.14.2 Toll-like Receptors 1, 2 and 6

Both TLR1 and TLR6 are associated with TLR2 (Figure 2.5). The principal ligand for TLR1 is lipopeptide while for the TLR6 are lipoteichoic acid and lipopeptides. The main cytokines that are secreted by macrophages and dendritic cells after detection of the ligands by TLR1/6 are IFN-α, TNFα, and IL-1β (Veckman, 2007; Opitz *et al*., 2010). Lipoteichoic acid and peptidoglycan are major components of Gram-positive bacteria. These serve as PAMPs recognised by TLR2 (Philpott and Girardin, 2004). TLR2 stimulation on macrophages and dendritic cells secrete pro-inflammatory cytokines IL-1, IL-6, IL-12, TNF-α (Opitz *et al*., 2010) and type I interferon which plays a significant role in fighting viral infection (Yu and Levine, 2011). TLR2 also detects ligands such as lipoproteins and lipopeptides due to its ability to form heterodimers with TLR1 or TLR6 because they are all structurally related (de Oliviera Nascimento *et al*., 2012; Takeda and Akira, 2005) as shown in Figure 2.6.

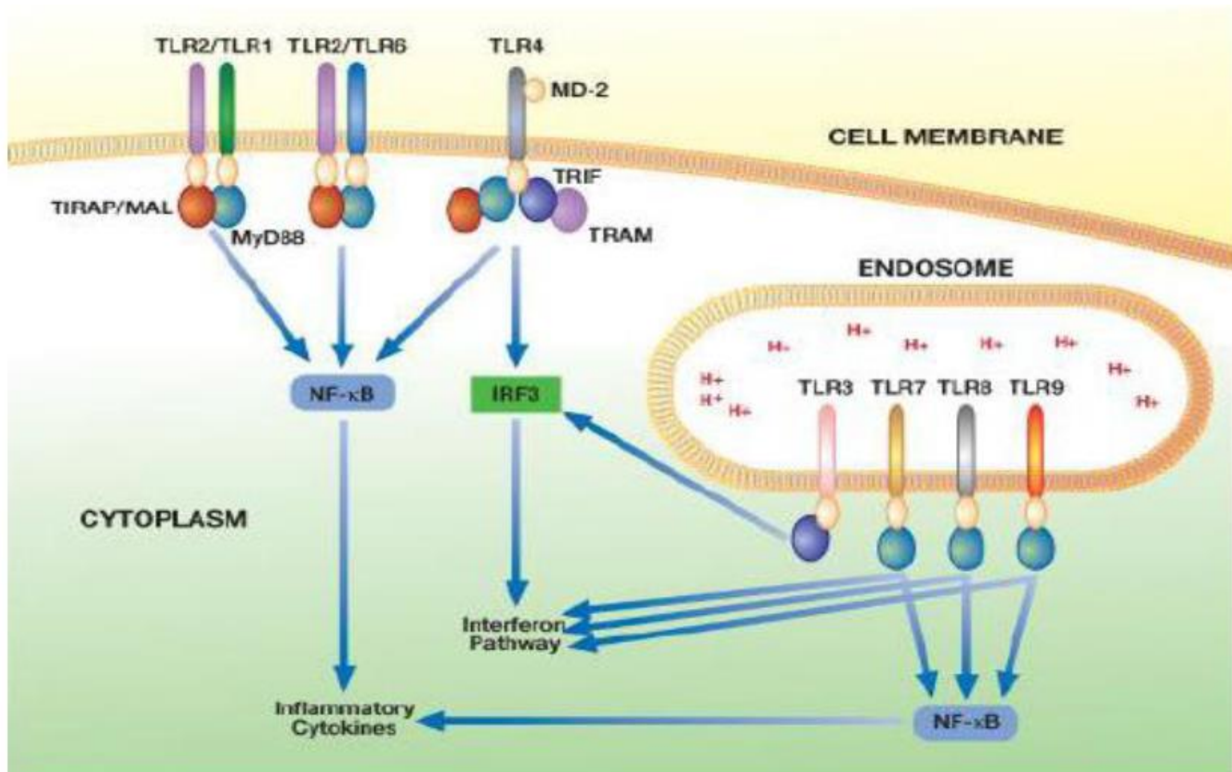


Figure 2.6: Toll-like receptors 1, 2 and 6 and their interaction proteins in humans (Source: Boehme and Compton, 2004).

2.14.3 Toll-like Receptor 3

When RNA viruses replicate inside cells, there is stimulation of immune cells due to the generation of double-stranded RNA (dsRNA) which activates protein kinase (Alexopoulou *et al*., 2001; Dunlevy *et al*., 2010) followed by activation of NF-κB which binds to the response element in the nucleus resulting in transcription and then translation leading to the production of inflammatory cytokines such as IFN-γ, TNF-α, and IL-6 (Alexopoulou *et al*., 2001). TLR3 plays a significant role in recognition of dsRNA and defending against RNA viral infection (Jelinek *et al*., 2011; Alexopoulou *et al*., 2001).

2.14.4 Toll-like Receptor 4

Lipopolysaccharide is an essential component of Gram-negative bacteria, and it is released when the bacterium is lysed. In the detection of bacterial lipopolysaccharide, CD14 acts as a co-receptor together with TLR4 and Myeloid Differentiation factor 2 (MD-2) proteins. For CD14 to bind LPS, lipopolysaccharide-binding protein (LBP) must be present (Kitchens, 2000; Vesy *et al.,* 2000). LPS is a ligand for TLR4, and after immune system activation of macrophages and dendritic cells the major cytokines that are secreted are IL-6, TNF-α, IL-2 and IL-1 (Takeda and Akira, 2005).

2.14.5 Toll-like Receptor 5

Flagellin is a major protein component of flagella, which are whip-like appendages found on the Gram-negative bacteria. These enable organisms to move through their environment. Flagellin

leads to secretion of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 due to its potent immune stimulatory activity and bacteria with flagella induce NF-κB activation after TLR5 detects the flagella ligand (Hayashi *et al*., 2001).

2.14.6 Toll-like Receptors 7 and 8

Recognition of single-stranded viral RNA by TLR7 and TLR8 in endosomal or lysosomal compartment leads to the activation of macrophages and dendritic cells resulting in the secretion of inflammatory cytokines. The main cytokines that are produced are IL-6, TNF-α, IL-10, IL-1β (Kattah *et al*., 2008).

2.14.7 Toll-like Receptor 9

Unmethylated CpG-DNA is found in bacterial and viral genomes while methylated CpG-DNA is found in eukaryotic cells. Methylated CpG-DNA does not stimulate immune cells whereas unmethylated CpG-DNA from bacteria and viruses is a potent stimulator of immune cells resulting in stimulation of dendritic cells, macrophages and B cell proliferation. These cells upon stimulation secrete pro-inflammatory cytokines TNF-α, IL-6 and IL-10, an anti-inflammatory cytokine. The unmethylated CpG-DNA is recognized by TLR9 (Häcker *et al*., 1998).

2.15 Signalling Pathways of Toll-like Receptors

2.15.1 Adaptor Proteins and Signal Transduction Pathway of Toll-like Receptors

Toll-like receptors need to recruit adaptor proteins from the cytoplasm some of which are Toll-IL-1R domain-containing adaptor proteins. These induce IFN-β-related Adaptor Molecule (TRAM), Toll-interleukin 1 Receptor (TIR) Domain-containing Adapter Protein (TIRAP), Myeloid Differentiation Primary Response gene 88 (MyD88) and TIR-domain-containing Adapter-

inducing Interferon-β (TRIF) (Yamamoto *et al*., 2003; Shigeoka *et al.,* 2007). The Inhibitory Kappa B Kinase (IKK) phosphorylates the Inhibitor kappa B (IκB) releasing Nuclear Factor kappa B (NF-κB), which with the help of transforming growth factor β Activated Kinase-1 (TAK1), a key regulator of signal transduction cascade. The activated NF-κB moves into the nucleus where it regulates immunological gene expression. In the nucleus, NF-κB binds to DNA sequence (5' GGGACTTTCC-3') at κB site (Urban and Baeuerle, 1990; Reimers *et al.,* 2005) and the transcription process is initiated resulting in the synthesis of mRNA by RNA polymerase enzyme. This is followed by the process of translation producing proteins and the effect changes in cell function such as the production of cytokines in response to stimuli.

2.15.2 Myeloid Differentiation Primary Response gene 88-dependent Pathway

Every TLR except TLR3 utilizes the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway response, and the main result is activation of NF-κB as well as the protein kinase (Figure 3.3). Conformational changes in the receptor upon ligand binding results in the recruitment of the adaptor protein MyD88, one of the TIR protein families (Shigeoka *et al*., 2007). MyD88 through other proteins recruits transforming growth factor beta-activated kinase-1 (TAK1) and then binds to I-kappa-B kinase beta (IKKβ) resulting in the phosphorylation of inhibitor of I-kappa B (IκB) allowing NF-κB to diffuse into the cell nucleus (Kawai and Akira, 2007). The NF-κB in the nucleus then binds to a specific upstream DNA sequence called the response element such as cyclic adenosine monophosphate (cAMP) response element (Kawai and Akira, 2004). Other proteins including co-activators and RNA polymerase are recruited to the DNA/NF-κB complex. The next process is activation of the transcription of defense related genes including inflammatory cytokines. The resulting defence proteins are secreted.

2.15.3 Toll -interleukin Receptor-domain-containing Adapter-inducing Interferon-β (TRIF) - Dependent Pathway

All known TLRs in humans use the MyD88 pathway except for TLR3 which uses the TRIF-dependent pathway (Kawai and Akira, 2004) signalling of TLRs via Myd88). Upon activation of TLR3, the TRIF adaptor is recruited resulting in the activation of kinases. Inflammatory Regulatory Factor 3 (IRF3) is then phosphorylated by TRIF/kinases signalling complex and translocated into the nucleus leading to the production of type I interferon (Kawai and Akira, 2004) and pro-inflammatory cytokines. The signaling of surface TLRs via MyD88 and endosomal TLRs via TRIF and MyD88 as shown in Figures 2.7 and 2.8 respectively.

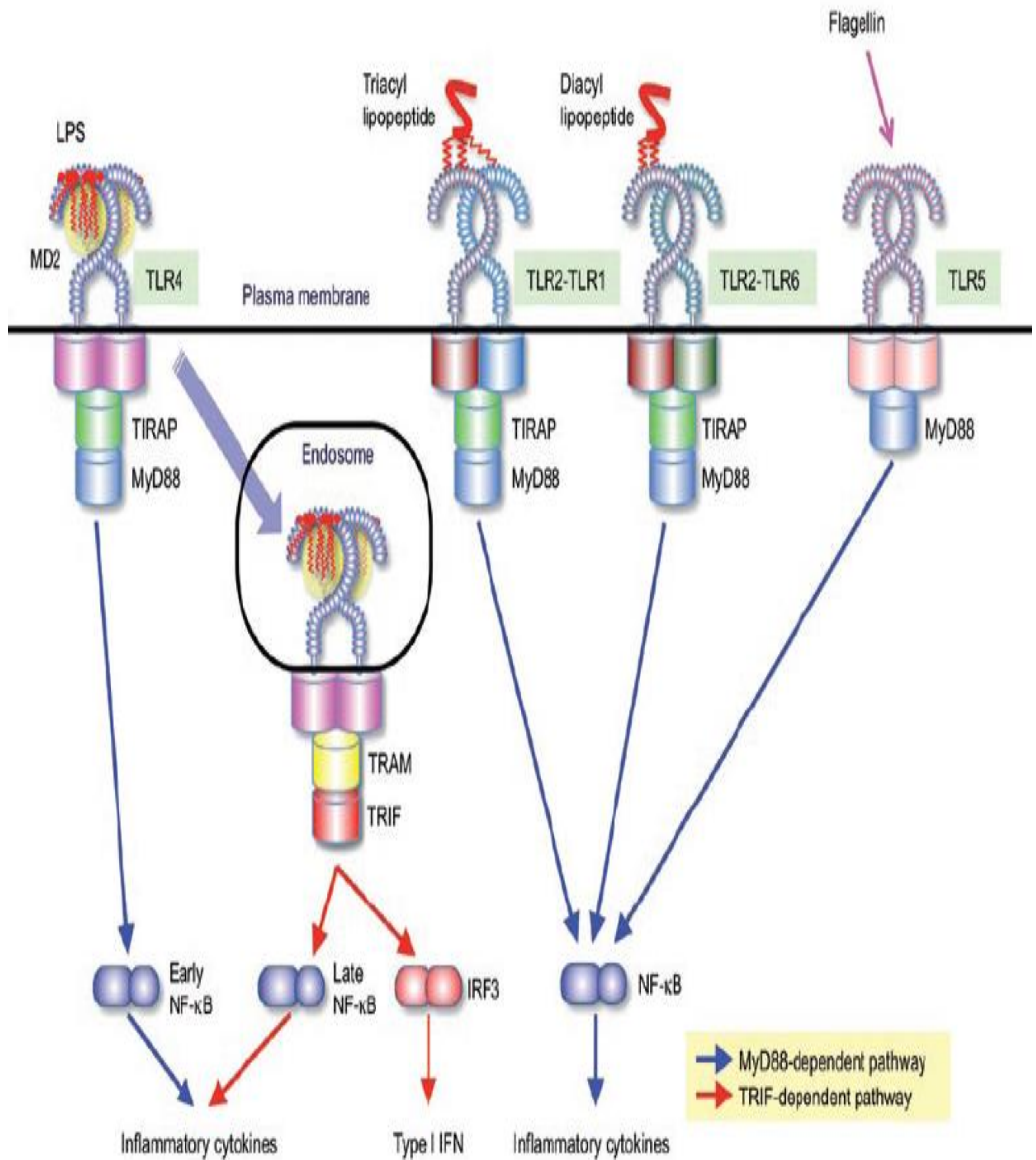


Figure 2.7: Signalling of surface TLRs via MyD88 (source: Kawai and Akira, 2010).

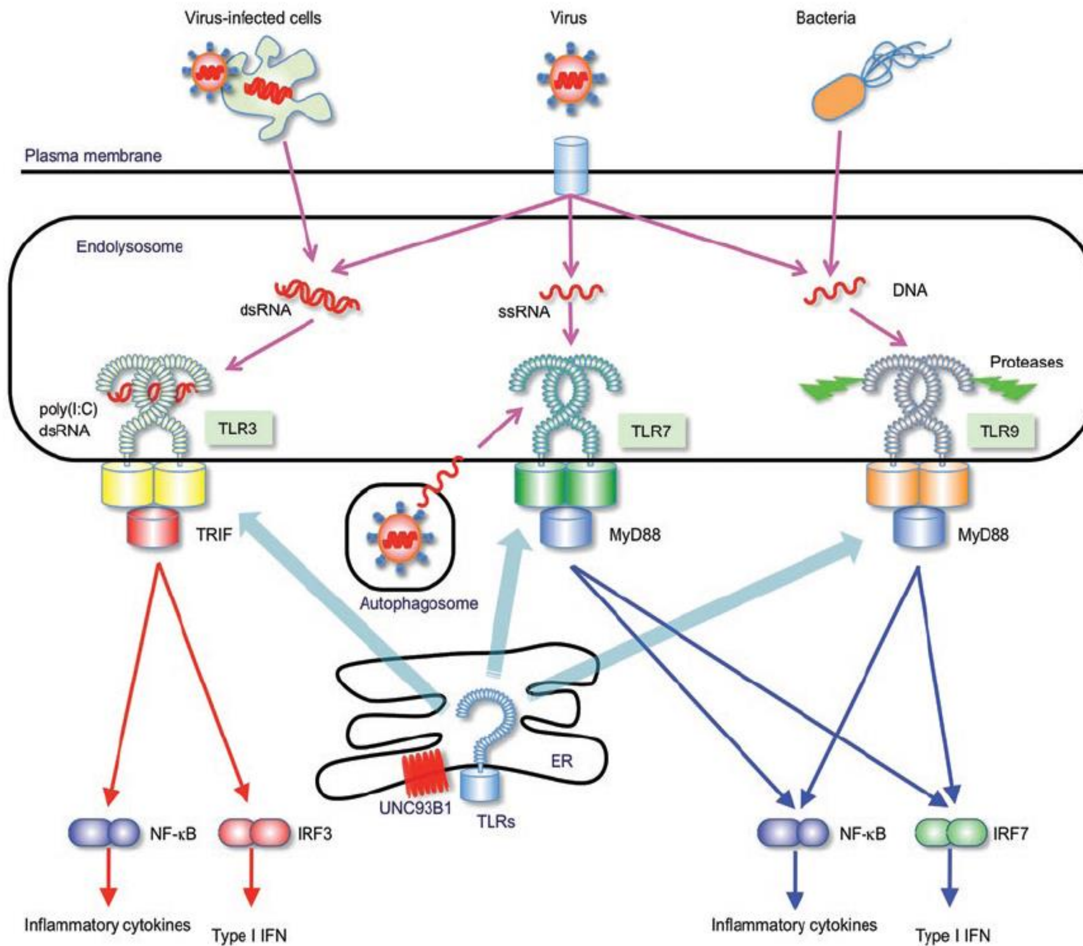


Figure 2.8: Signalling of endosomal TLRs via TRIF and MyD88 (Source: Kawai and Akira, 2010).

2.15.4 Toll–interleukin 1 Receptor (TIR) Domain–Containing Adaptor Protein (TIRAP)-Dependent Pathway

Studies have shown that adaptor protein MyD88 is the primary signalling pathway through which conserved microbial products are recognised by TLRs (Kawai and Akira, 2010). However, it has been shown that in MyD88-deficient mice, TLR4 can activate NF-κB and recognise its ligand LPS suggesting that another adaptor protein exists to mediate signalling independent of adaptor protein MyD88 (Horng *et al.*, 2001). A Toll-Interleukin I receptor (TIR) domain-containing adaptor

protein (TIRAP) was identified shown to control signalling pathway in the absence of MyD88. The primary function of TIRAP also called Mal is to facilitate the signalling as an adaptor protein to TLR4 (Sheedy and O'Neill, 2007).

2.15.5 Tumour Necrosis Factor Receptor-Associated Factor (TRAF) -Dependent Pathway

Most TLRs members use MyD88 as an adaptor protein which has binding sites that overlap with those of MyD88-adapter-like (Mal). Mal as a sorting adaptor for MyD88 works in combination with TRAM as well as IL-1/IL18 receptors. It has also been experimentally demonstrated that TRAM is an adaptor for both TLR4 and TLR2/6 signalling (Sacre *et al*., 2007). A summary of the PAMPs that have been shown or hypothesised to interact with PRRs is given in Table 2.1 together with the respective adaptor proteins through which they use.

Table 2.1: Different PAMPs involved in microbial translocation and their respective adaptor proteins

PAMP	Origin	TLR	PAMP location	Adaptor protein	References
Lipopeptides	GPB	TLR1	Cell surface	MyD88	Akira *et al*., 2006
Peptidoglycan Lipoteichoic, lipoprotein	GPB/GNB	TLR2	Cell surface	MyD88, Tram	Dunlevy *et al*., 2000
Double-stranded RNA	RNA viruses	TLR3	Endosome	TRIF	Jelinek *et al*., 2011
Lipopolysaccharide	GNB	TLR4	Cell surface	MyD88 TIRAP (Mal)	Vesy *et al.,* 2000; Tekeda and Akira 2007
Flagellin	GNB	TLR5	Cell surface	MyD88	Kattah *et al.,* 2008
Lipoteichoicacid, lipopeptides	GPB/GNB	TLR6	Cell surface	MyD88	Takeda and Akira, 2007
Single stranded RNA	RNA viruses	TLR7	Endosome	MyD88	Kattah *et al*., 2008
Single stranded RNA	RNA viruses	TLR8	Endosome	MyD88	Kattah *et al*., 2008
Unmethylated CpG-DNA /16S rRNA	bacterial DNA	TLR9	Endosome	MyD88	Takeda and Akira, 2007

PAMP, Pathogen-Associated Molecular Pattern; GPB, Gram positive bacteria; GNB, Gram negative bacteria; TLR, toll-like receptor; MyD88, Myeloid differentiation primary response gene 88; TRAM, Toll- interleukin 1 receptor-domain-containing adapter-inducing interferon-β; TIRAP, Toll–interleukin 1 receptor domain–containing adapter protein; TRIF, Toll- interleukin 1 receptor -domain-containing adapter-inducing interferon-β

2.16 Mechanisms of Microbial Translocation

At present, there are two possible pathways of gastrointestinal permeability through which microbial translocation is thought to occur (Barbara *et al*., 2005). These are the transcellular and the paracellular pathways. The transcellular pathway is through the enterocytes and involves the

use of the membrane pumps and certain channel proteins while the paracellular pathway is between cells, either as a result of opening up of tight junctions or damage to extracellular matrix proteins (MacFie, 2004). The effects of microbial translocation are numerous and include defects in the intestinal barrier, changes in the local immune defences and increases in the concentration of some microbial factors (Guarner and Soriano, 2005). Under normal circumstances, the intestinal barrier is an intact layer of one epithelial cell system, and these cells are close to each other forming the tight junctions (Turner, 2009). These tight junctions are held together by a family of proteins called claudins which do interact in a more tissue-specific way and result in charge-selective and size-selective barriers (Zeissig *et al*., 2007) as shown in Figure 2.9.

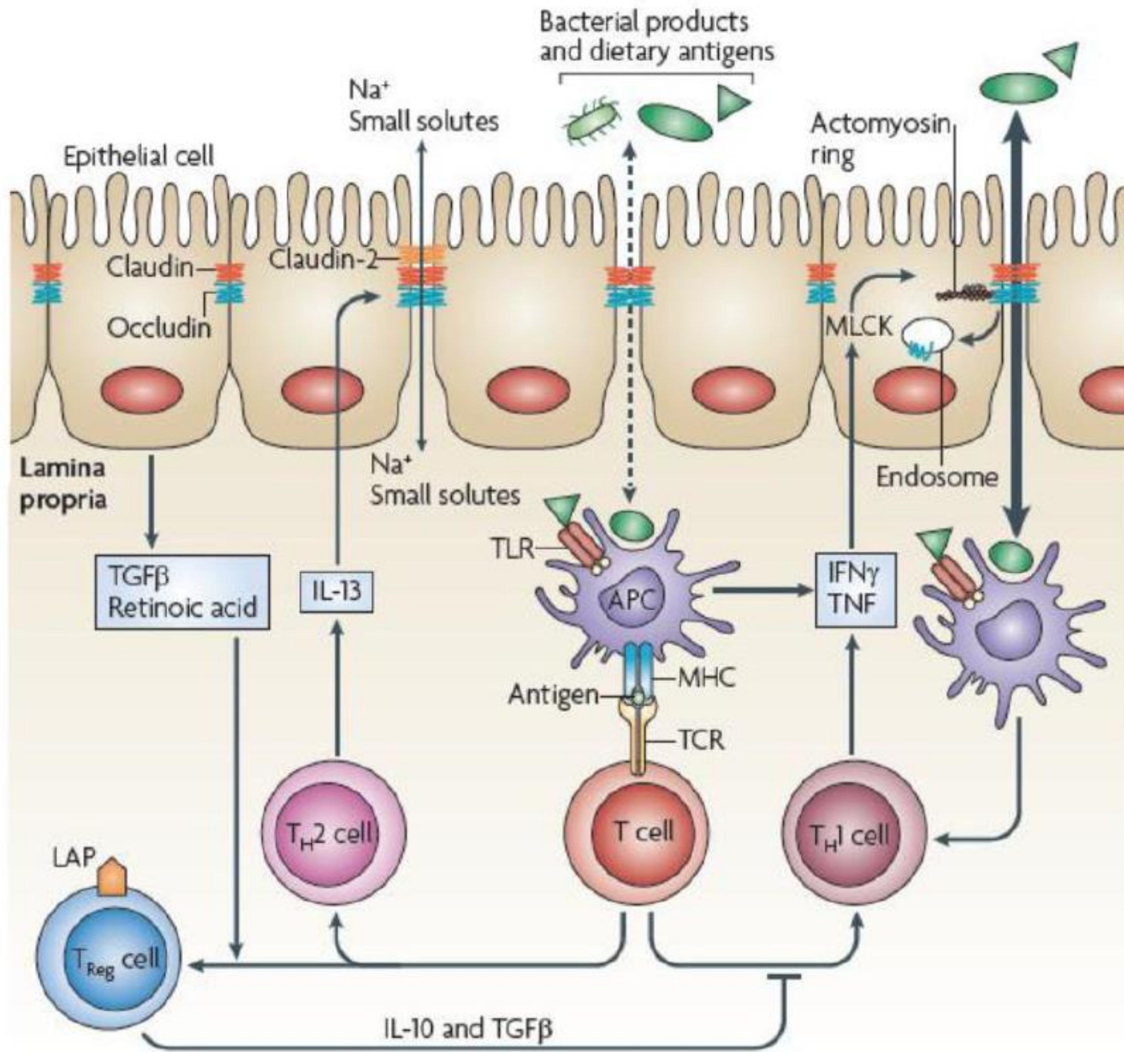


Figure 2.9: The epithelium and tight junction as integrators of mucosal homeostasis (Source: Turner, 2009).

The tight junctions are the major barrier between the cells (paracellular) in the intestinal mucosa. They in some ways determine the movement of ions and other molecules. The tight junctions are complicated and have at least two mechanisms by which particles pass through them namely a pore pathway and a leak pathway (Turner, 2009). Recent studies have shown that claudins are very vital in the pore pathway while ZO-1 (Shen *et al*., 2011) which separates apical and basolateral plasma membrane domains and occludins play a significant role in the leak pathway. The control

of these pathways involves a lot of other proteins and a complicated mechanism which involves both intracellular and extracellular signalling pathways. Studies have shown that LPS and TNF-α can induce the signalling event leading to the activation and phosphorylation of myosin II regulatory light chain (MLC) by myosin II regulatory light chain kinase (Watson et al., 2005; Clayburgh *et al*., 2005) inducing barrier dysfunction. Under normal circumstances, the molecules which do not pass through the intestinal mucosa barrier into the portal circulation to cross due to ‘leakiness' which has been found to be bi-directional (Figure 2.8) because even serum molecules can cross and get into the gut lumen (Pelaseyed *et al*., 2014). Biopsy samples are used to show reductions in small intestinal villi height, blunting and atrophy, increased numbers of intraepithelial lymphocytes, and a lot of T-cells infiltrate the underlying lamina propria by T-cells (Kau *et al*., 2011).

2.17 Direct Biomarkers of Microbial Translocation

2.17.1 Lipopolysaccharide as a Direct Biomarker of Microbial Translocation

Lipopolysaccharide (LPS) is a major component of the Gram-negative cell wall, highly antigenic and a ligand for TLR4. Since humans do not have LPS, its presence in blood is considered as a direct biomarker of microbial translocation (Marchetti *et al*., 2013; Fukui, 2016; Bellot *et al*., 2013). LPS is believed to cross the compromised intestinal epithelial barrier from the luminal side into the MLN or systemic circulation and has been reported in health conditions associated with compromised barrier function. In Northeastern Brazil, Children with EE and functional intestinal barrier disruption had significantly higher levels of LPS which was associated with stunting (Guerrant *et al*., 2016). The investigators concluded that the finding could be important for effective intervention in children with enteropathy in impoverished settings. Similar results were found in Zimbabwean children who were stunted and possibly with EE. Some of these reported

cases of stunting were associated with other indirect biomarkers including CRP and sCD14 compared with controls (Prendergast *et al*., 2014).

A study in Zambian adults with EE showed that their plasma LPS strongly correlated with cell shedding on endoscopy and elevated levels of plasma fatty acid binding protein (FABP), a plasma biomarker of epithelial damage. The findings suggested that damaged intestinal epithelium in EE lead to the leakage of gut contents into the bloodstream resulting in increased plasma circulating biomarkers (Kelly *et al*., 2016). The T-cell dependent B-cells produce endogenous endotoxin-core antibodies (EndoCab), naturally existing antibodies, in response to exposure to LPS. A study of children with EE in the Gambia found that serum EndoCab were elevated and associated with growth faltering, reduced weight growth and increased intestinal permeability measured by L: M ratio (Lunn *et al*., 1991). In a case-control study conducted in Zambia in HSS patients and healthy controls, there was no difference in LPS that was observed. However, from the same study, indirect biomarkers of microbial translocation such as sCD14 and CD163 were higher in HSS patients compared with healthy controls (Sinkala *et al*., 2016) suggesting microbial translocation in HSS patients. In a study of the mouse model, LPS levels were found to be higher in mice with EE compared with healthy controls (Brown *et al*., 2015) suggesting more microbial translocation in mice with EE. In chronically infected populations, some studies have reported elevated biomarkers of microbial translocation such as LPS. In a South African study, LPS was reported to be higher in chronic HIV patients compared with HIV negative individuals. The results also showed significant correlation with others biomarkers such as sCD14 and TNF-α (Cassol *et al*., 2010) elevated LPS in HIV patients have been reported elsewhere (Brenchley *et al*., 2006; Jiang *et al*., 2009) suggesting microbial translocation.

2.17.2 16S rRNA as Direct Biomarker of Microbial Translocation

Bacteria present in blood have traditionally been detected using culture-dependent methods. While microbiological approaches have been successful at detecting the bacterial presence, they have been criticised for being less sensitive (Matsuda *et al*., 2007; Clifford *et al.*, 2012). The main drawbacks for blood culture technique include longer turn-around time between blood sampling and use of results by clinicians (usually days), difficulties in detecting fastidious microorganisms, need for bacteriological know-how and increased personnel workload (Lagu *et al*., 2012). Culture technique is associated with problems and to overcome them molecular methods can be used which are more sensitive, specific, and efficient and less time-consuming. Progress has been made in the development of culture-independent molecular techniques to detect bacteria in blood at the molecular level, and 16S rRNA is usually used (Han *et al*., 2012). Bacterial 16S rRNA is a component of the 30S small subunit of a prokaryotic ribosome (Schluenzen *et al*., 2000) serving several functions including as scaffold molecules for ribosomes, involvement in protein synthesis through the pairing of the codons and anticodons (Langille *et al*., 2013; Jay and Inskeep, 2015). The genes in the 16S rRNA region are slow to evolve. In certain regions, they are highly conserved, and for these reasons, 16S rRNA genes are routinely used to detect bacteria by the use of specifically designed primers which detect certain conserved regions of the 16S rRNA structure (Kramski *et al*., 2011) as shown below (Figure 2.10).

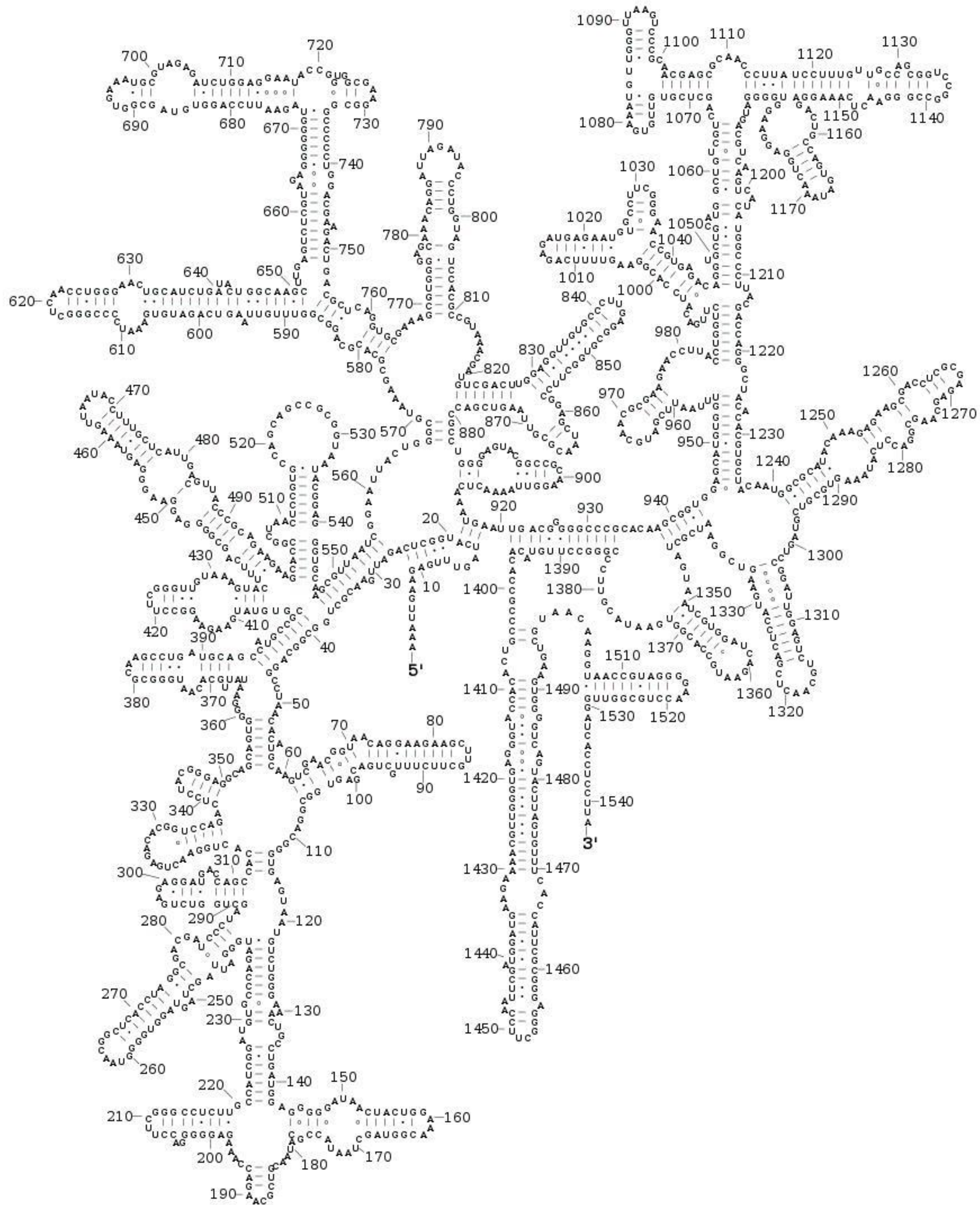


Figure 2.10: Structure of 16S rRNA gene based on Escherichia coli (Source: Yang et al., 2016).

Since the 16S rRNA or the genes which encode them are only present in bacteria, their presence in human blood is considered to be indicative of exposure to bacteria. To quantify bacterial 16S rRNA gene copy number, bacterial DNA must be isolated from blood. Many studies have used this approach in many conditions with the compromised gut barrier (Brown *et al*., 2015; Jiang *et al*., 2009). In a Malawian study, using 16S rRNA sequence, children with EE were found to have more and diverse bacteria than those without EE. In a mouse model of EE, levels of 16S rRNA genes in plasma and MLNs were significantly higher in mice with EE compared with those without EE suggesting more microbial translocation in mice with EE due to the compromised intestinal gut barrier (Brown *et al*., 2015).

In the USA, a cross-sectional study was conducted to compare plasma I6S rRNA copy number among HIV-positive treatment naïve patients and HIV-negative individuals. HIV-positive treatment naïve patients had significantly higher copy number than HIV-negative individuals, and when a HAART-treated group was analysed, there was a positive correlation with LPS as well as other biomarkers of immune activation (Jiang *et al*., 2009). In a cross-sectional study, plasma 16S rRNA gene was reported to be higher in HIV-positive immunologic-non-responders compared with HIV-positive responders to HAART treatment, and they showed that plasma 16S rRNA was associated with LPS in immunologic-nonresponders. It was concluded that the correlation enhanced the systemic translocation of microbial products (Marchetti *et al*., 2008). In a longitudinal study, HIV-infected patients starting HAART with the CD4 count of less than 200cells/µl were analysed and compared with patients with CD4 count ≥250 cells/µl. Both groups were found to have elevated plasma 16S rRNA but with no significant differences in the levels between the groups. Plasma 16s rRNA analysis revealed the presence different bacterial species

before and after treatment in both groups with evidence also drawn from LPS and soluble CD14 comparisons (Merlini *et al*., 2011). In a prospective longitudinal study, plasma 16S rRNA copy numbers were measured at baseline and 6 months and compared with chronic untreated HIV infected patients. The baseline results and those obtained at six months after the commencement of the study were similar when compared, but both were significantly lower than those found in patients with chronic untreated HIV infection suggesting the occurrence of microbial translocation in the chronic untreated HIV group (Chevalier *et al*., 2013). On the contrary, a study compared plasma 16S rRNA copy number of HIV infected adults at various stages of the disease. Patients were categorised as long-term non-progressors, treatment-naive patients with CD4+ cell counts of greater than 350 but less than 500 cells/ μL and those with the CD4+ cell count of <100 cells/μL who after receiving effective treatment achieved a CD4+ cell count of 1350 cells/mL. Others were those with the CD4+ cell count of <100 cells/μL who after receiving >2 years of effective treatment did not achieve a CD4+ cell count of >200 cells/μL and healthy uninfected individuals. The results showed no significant differences between HIV-negative group and each one of the 4 HIV-positive groups (Ferri *et al*., 2010) suggesting no difference in microbial translocation among the different groups. Studies have reported the presence of substantial amount of 16S rRNA in plasma of healthy individuals whose origin still is unknown (Nikkari *et al*., 2001).

In a similar study, 16S rRNA gene was analysed in healthy individuals using real-time PCR (Moriyama *et al*., 2008) and of the detected bacteria none belonged to the indigenous gastrointestinal flora which left the conclusion not clear as to whether these could have been of intestinal origin. Detection of bacteria in blood using molecular techniques has continued to grow in importance owing to their promptness and sensitivity when compared with standard bacterial culture methods. Quantitative real-time PCR is one such method (Pletz *et al*., 2011). Quantitative

PCR is a method for detection of bacteria associated with microbial translocation, and its advantages include speed and sensitivity of detection chemistries such as SYBR green (Jiang *et al*., 2009; Ferri *et al*., 2010).

One limitation is that Taq DNA polymerase and real-time PCR master mix have been reported in some cases to contain bacterial DNA (16S rRNA) which successfully can be amplified resulting in false-positive reactions (Hughes *et al*., 1994). Some of the methods have been used to reduce contamination including ultraviolet irradiation and DNase I treatment of samples. The challenge has been that these methods tend to affect the sensitivity of real-time PCR but not conventional PCR (Corless *et al*., 2000). Broad range real-time PCR amplification using 2 x SYBR green master mix as a fluorescence signal can be employed without sensitivity being affected (Tseng *et al*., 2003) if treatment with DNase I at 37 °C for 60 min. Treatment of real-time PCR master mix before PCR reaction significantly minimises bacterial DNA contamination of up to 500 fg and is essential for triumphant improvement of broad-range rRNA real-time PCR. Selected details of oligonucleotide 16S rRNA primers that have been used by various research groups in quantitative real-time PCR are given below (Table 2.2).

Table 2.2: Summary of the list of 16S rRNA primers from selected research groups

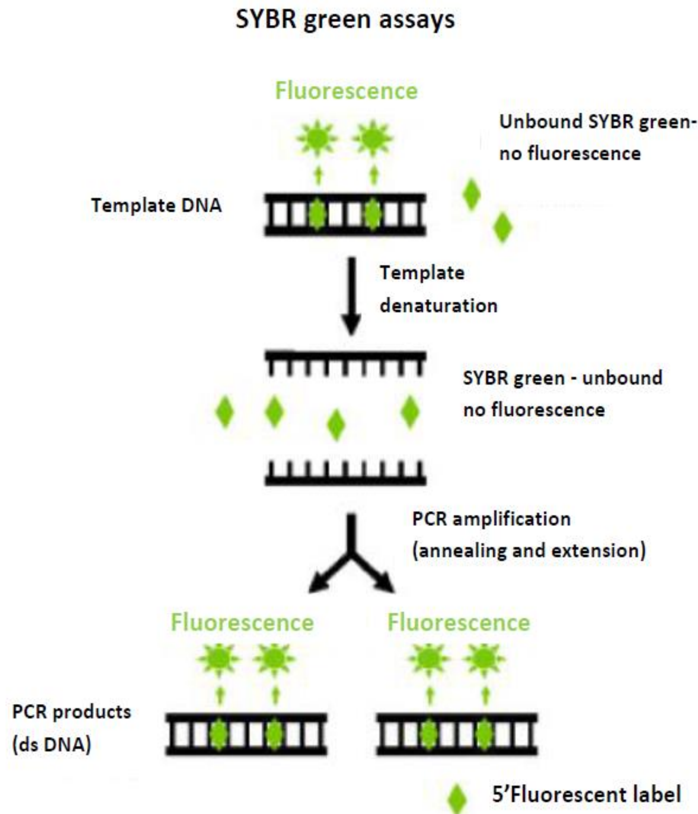
PRIMER	SEQUENCE (5' – 3')	POSITION	REFERENCE
16S - F8	AGTTTGATCCTGGCTCAG	8 - 25	Chen *et al*., 2012
16S - F27*	AGAGTTTGATCMTGGCTCAG	8 - 27	Youssef *et al*., 2009
16S - F519	CAGCAGCCGCGGTAATAC	517 - 534	Foley *et al*., 2006
16S - R515*	GWATTACCGCGGCKGCTG	515 - 498	Hill *et al*., 2009
16S - R519	TTACCGCGGCTGGCA	519 - 505	Kotetishvili *et al*., 2002
16S - R785	TGGACTACCAGGGTATCTAATCC	785 - 763	Kaltenpoth *et al*., 2011

* where W = A or T, M = A or C, K = G or T

2.17.2.1 Optimizing Real-time PCR using SYBR green

SYBR green is an intercalating dye that binds to double-stranded DNA. Sometimes it can generate a higher fluorescence signal than expected due to non-specific product formation and over-estimation of target template quantity. In setting up SYBR green quantitative PCR for the detection of 16S rRNA gene, detailed and careful optimisation of PCR assay is required to guarantee that the only fluorescence signal is from the target product. The choice of primers is cardinal to avoid primer-dimer formation through self-complementarity. The particular product, 16S rRNA gene of quantitative real-time PCR can be detected by conducting melting curve analysis to confirm the identity of the amplicon. During melting curve analysis the 16S rRNA product is heated, as the temperature increases, it reaches the melting point of the product and the two DNA strands separate. The SYBR green in between the double-stranded DNA dissociates leading to decrease in the fluorescence signal at the dissociation temperature 50% of the of the product is denatured and confirming that targeted template is present (Gonzalez-Escalona *et al*., 2006) as illustrated below (Figure 2.11).

In quantitative real-time PCR (q-PCR) amplification, a curve can be segregated in four phases which are background noise (baseline), exponential, linear and plateau. In real-PCR, the initial numbers of target template are directly proportional to amplicons detected in the sample, and this circumvents problems that are associated with endpoint PCR where products (amplicons) are only analysed at the end of the assay. Due to being fluorescence-based technique, q-PCR has the wider dynamic range as it is capable of discriminating a two-fold change in target concentration and not endpoint PCR (Smith and Osborn, 2008). Illustration of real-time PCR using SYBR Green is shown below (Figure 2.11).



During real-time PCR, SYBR green binds to anything double stranded DNA and give out fluorescence signal but in unbound state, SYBR green does not fluorescent.

Figure 2.11: Illustration of SYBR Green assay (source: Federation of European Microbiological societies, 2008).

2.17.2.2 PCR Efficiency

The recommended efficiency range of real-time PCR is from 90% to 110% (Applied Biosystems) due to standard deviation in dilution series, and for a PCR with 100% ±10% efficiency a slop of -3.3 ± 10 % is obtained. Some factors that the efficiency of PCR depends on include sample quality, assay, and master mix performance. The sensitivity of a PCR is determined by its efficiency and every time there is a change in the reagents or machine these parameters have to be evaluated.

2.17.2.3 R^2 Value

In evaluating PCR efficiency, R^2 is also important in statistical terms as it shows how good the value is at estimating another. The R^2 ranges from 0 to 1 being the maximum. On the Y-axis (Ct) the closer the R^2 value is to 1, the better the accuracy of predicting the value of X (X-axis). On the other hand, if R^2 is zero it cannot be used to predict the value of Y. So the closer the value of R^2 to 1, the better it provides a correlation between two values (Smith and Osborn, 2008).

2.17.2.4 PCR Sensitivity

In a PCR technique amplification of one copy of initial template means the final level of sensitivity have been reached even without considering the cycle threshold value. The sensitivity of a PCR partly depends on efficiency. To successfully set up a suitable PCR and determine its performance, efficiency, R^2, specificity, sensitivity as well as non-template control (NTC) Ct value must be evaluated. The model of q-PCR run (100% efficiency) has R^2 of 100% and a slope of -3.3 indicating that every q-PCR cycle, indicating that the template in the sample is doubling at every cycle.

2.17.3 Toll-like Receptor Ligands as Direct Biomarkers of Microbial Translocation

The RAW-blue mouse macrophage cell line expresses Toll-like Receptors (TLRs) 1-9, but not TLR5. The receptors detect PAMPs and trigger signalling transduction cascades leading to the activation of NF-κB, a transcription factor. As the cell line has been modified by the inclusion of a Secreted Embryonic Alkaline Phosphatase (SEAP) gene under the control of an NF-κB promoter, the ligand binding to all TLRs can be quantified by measuring SEAP colorimetrically.

The detection system therefore quantifies the total PAMPs in the sample. The Mechanism of NF-κB activation after stimulation of TLRa is shown in Figure 2.12.

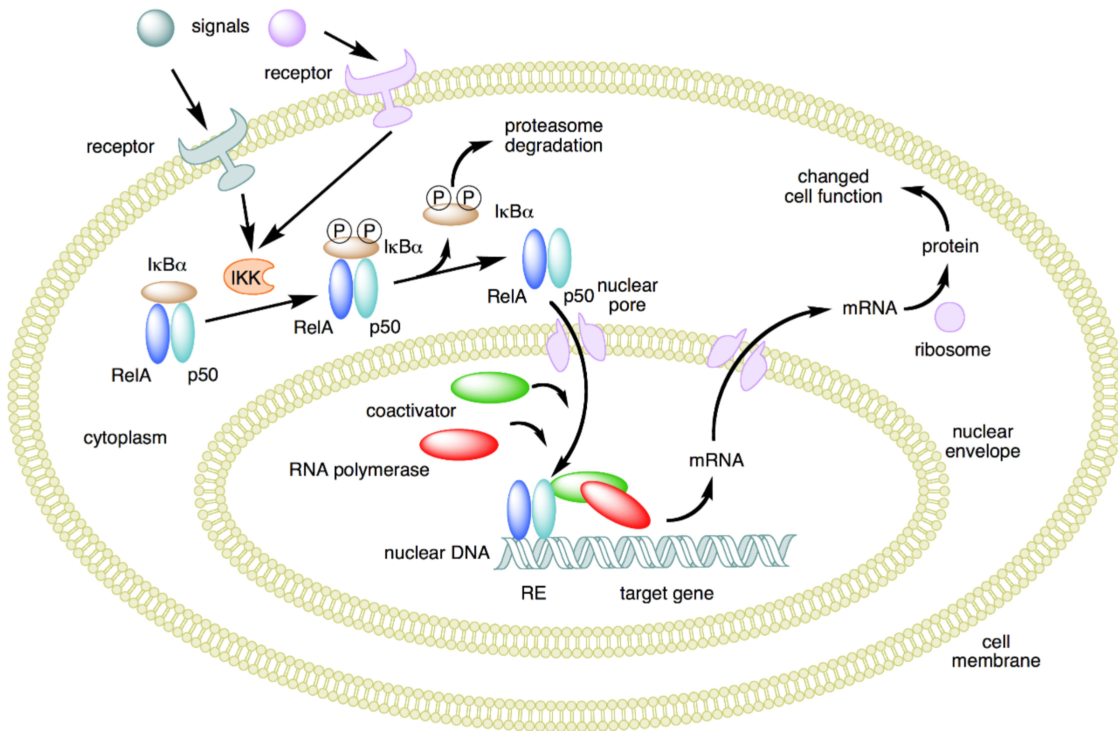


Figure 2.12: Mechanism of NF-κB activation after stimulation of Toll-like recepter (Source: Braser, 2006).

Many *in-vitro* experimental studies have been conducted where RAW-Blue cells have been stimulated with different ligands and SEAP has been measured in the cell culture supernatant. In one example, RAW-Blue macrophages were stimulated with 1mg/ml of LPS cultured in Dulbecco Modified Eagle Medium (DMEM) at 37°C for 24 hours under 95% O_2 and 5% CO_2 in a humid

atmosphere. The study showed that the cell line was activated as detected by NF-κB. There was also an increase of SEAP which correlated with TNF-α, IL-10 and IL-1β produced suggesting immune activation (Chen *et al*., 2014). In another study, RAW-Blue macrophages were cultured in similar conditions and stimulated with different LPS concentrations ranging from 5 to 40μg/ml. All concentrations stimulated the cells as measured by NF-κB and TNF-α, IL-6 and IL-1β that were detected in the culture medium (Soromou *et al*., 2012). In another study, Agrawal *et al*., 2013 stimulated TLR2 from the RAW-Blue cells with its specific ligand, peptidoglycan as well as TLR4 ligand LPS. They found that both ligands stimulated their respective receptors with the production of IL-1β and TNF-α detected by expression of their respective mRNAs using western blot. Many other studies have reported the use of LPS to stimulate RAW-Blue mouse macrophages line and measured immune activation by detection of both pro-inflammatory and anti-inflammatory cytokines in the cell culture supernatant (Szliszka *et al*., 2013; Choi *et al*., 2014; Bueno-Silva *et al*., 2015).

2.18 Biomarkers of Host Immune Response to Microbial Translocation

2.18.1 Lipopolysaccharide-binding Protein as a Biomarker of Host Immune Response to Microbial Translocation

Lipopolysaccharide Binding Protein (LBP) is a soluble acute-phase protein that binds bacterial LPS and interacts with CD14 and myeloid differentiation factor 2 (MD-2). This complex binds to Toll-like receptor 4 (TLR4) to initiate an immune activation. In mice, studies have shown that the protein is not essential for LPS clearance from the circulatory system but needed for speedy acute-phase protein response so that the immune response can be elicited (Muta and Takeshige, 2001). In mice model, LBP knockout mice were significantly more susceptible to bacterial infections

compared with healthy wild-type mice suggesting a deficiency in effective innate immune response (Knapp et al., 2003).

Since LBP is needed to bind LPS and is a surrogate biomarker of bacterial translocation, it has been suggested to be a more significant surrogate biomarker of bacterial translocation in patients with co-infection with HIV and HCV than both LPS and sCD14 (Nyström *et al*., 2015). In a study of a relationship between LBP and LPS in patients with severe sepsis and septic shock, patients had significantly higher endotoxin and LBP levels compared with the healthy controls. However, there was no correlation between LBP and endotoxin levels even though both LBP and endotoxin have been proposed to have a prognostic value in patients with severe sepsis (Opal and Esmon, 2002). Lack of correlation of LBP and LPS might be explained by a suggested mechanism of high LBP concentration believed to hinder LPS binding to monocytes and initiation of pro-inflammatory cytokines (Zweigner *et al*., 2001). But in a study of adult Zambians with EE, LBP correlated with LPS in participants who were HIV sero-positive but not in HIV sero-negative individuals (Kelly *et al*., 2016) suggesting that the correlation between LPS and LBP may be complex. More studies are needed in the different groups of people to understand the relationship between LBP and LPS. In patients with hepatosplenic schistosomiasis, plasma LBP was significantly higher compared with healthy controls suggesting microbial translocation in HSS patients (Sinkala *et al*., 2016).

2.18.2 Soluble CD14 as a Biomarker of Host Response to Microbial Translocation

CD14 is a target for a complex of LPS-LBP and an indirect biomarker of microbial translocation. Membrane CD14 (mCD14) is expressed on the surface of most innate immune cells but mainly on macrophages. In the presence of LPS, the expression of sCD14 is upregulated and act together

with MD2 and TLR4 to initiate clearance of LPS (Koutsounas *et al*., 2015). Guerrant *et al*., 2016 studied children with EE and suggested that increased levels of sCD14 were associated with increased intestinal permeability as measured by L: M ratio indicative of microbial translocation. In Bangladesh, a study was conducted in children with EE to determine whether host factors are associated with immune response to an Oral Cholera Vaccine (OCV). One of the measured plasma biomarkers was sCD14 which was found to be a positive predictor of the IgG response against LPS (Falkard *et al*., 2015). Similar results were reported by other investigators in the same country in the similar study population (Uddin *et al*., 2016). Many studies have shown that chronic HIV infection is associated with increased intestinal permeability and chronic immune activation (Jiang *et al*., 2009; Cassol *et al*., 2010). Chronic immune activation has been reported to be a better predictor of HIV disease progression than viral load (Brenchley *et al*., 2006). In a nested case-control study of chronic HIV patients, some plasma biomarkers of microbial translocation measured included sCD14 and were found to be a significant independent predictor of mortality. The study concluded that there were more microbial translocation events in the cases than in the control group probably due to increased permeability. It was also proposed that interventions should include attenuation of immune activation in chronic HIV infection in order to ameliorate the disease (Sandler *et al*., 2011).

In a study to investigate whether initiation of ART with or without co-trimoxazole prophylaxis reduces biomarkers of microbial translocation after 1 year of treatment, there was no change observed before and after treatment in sCD14 levels suggesting that treatment probably did not change microbial translocation (Vesterbacka *et al*., 2015). In many patients with cirrhosis regardless of the aetiology, sCD14 has been used as a biomarker of microbial translocation and is

believed to play a significant role in the liver disease prognosis (Kaser *et al*., 2002). In a study of patients with hepatitis B and C infections, sCD14 was found to be both a biomarker of microbial translocation and a good prognostic biomarker of the diseases (Sandler *et al*., 2011). Soluble CD14 has also been found to be a biomarker of microbial translocation in other diseases such as Crohn's disease (Lakatos *et al.,* 2011) and experimental colitis using animal model (Buchheister *et al*., 2017). In HSS infection, when sCD14 was compared to healthy controls, it was significantly elevated (Sinkala *et al*., 2016) suggesting that there was more microbial translocation in the HSS patients compared to healthy controls.

2.18.3 Soluble CD163 as a Biomarker of Host Response to Microbial Translocation

Soluble CD163 is a biomarker of Kupffer cell activation, and a macrophage secreted as sCD163 which is upregulated in inflammatory diseases (Jone *et al*., 2013). In a recent study, in adult Zambians with EE diagnosed by confocal endomicroscopy, sCD163 was not associated with LPS in either in those with HIV infection or those without the infection (Kelly *et al*., 2016). The finding did not support the hypothesis that HIV drives systemic inflammation and microbial translocation as suggested by others (Brenchley *et al*., 2006). Another study was conducted to compare plasma sCD163 as a biomarker of macrophage/monocyte activation between patients who were HIV-positive for more than 1 year and HIV negative individuals. HIV infected individuals had significantly elevated plasma sCD163 compared with the HIV-negative people suggestive of more microbial translocation in the former group (Burdo *et al*., 2011).

A longitudinal study was conducted in undernourished HIV infected adult Zambians initiating ART. After 3 months of treatment most biomarkers of microbial translocation and sCD163 did not improve. The study concluded that in HIV patients impaired intestinal immune defence plays a

role in contributing to innate immune activation (Canipe *et al.,* 2014), but it is also possible that 3 months was too short to observe the expected effect. Investigators in Sweden conducted a cohort study of 933 HIV infected patients and measured their plasma sCD163 and correlated with the progression of the disease. They showed that sCD163 was an independent predictor of mortality and suggested that it should be considered as a target for intervention (Knudsen *et al*., 2016). A recent study in Zambia of microbial translocation in HSS patients showed that sCD163 was significantly higher in these patients compared with healthy controls (Sinkala *et al*., 2016) suggesting that there was more Kupffer cell activation in response to microbial translocation in HSS patients. In marked enteropathy in an accelerated SIV/pigtailed macaque model of AIDS to examine microbial translocation, results showed that half the animals had histological evidence of compromised intestinal barrier. The results showed modest indication of microbial translocation as measured by sCD163, which was supported by other biomarkers such as LBP, 16S rRNA and sCD14 (Croteau *et al*., 2017).

2.24.4 C-Reactive Protein as a Biomarker of Host Response to Microbial Translocation

C-reactive protein (CRP) is a protein with a short half-life, produced by the liver and a non-specific biomarker of inflammation in the body (Thompson *et al*., 1999). In a study of 49 Zambian adults with EE identified by intestinal epithelial defects, plasma biomarkers CRP, sCD14, CD163, and LBP were all not associated with LPS, a direct biomarker of microbial translocation which was detectable in all samples (Kelly *et al*., 2016). A case-control study was conducted in Zimbabwe in 202 HIV-unexposed infants who were stunted, as defined by height-for-age Z-score (HAZ) less than -2 and compared with healthy controls with a Z-score of greater than - 0.5 HAZ both at 18 months. In that study, infants who were stunted showed significantly higher levels of CRP

compared with healthy controls suggesting that probably enteropathy begins early in life and chronic inflammation perhaps impair the growth of infants (Prendergast *et al*., 2014). In the same study, the finding was supported by results of other biomarkers such as Insulin-like Growth Factor 1 (IGF-1), a hormone that is responsible for linear growth, especially in infants. It was reported that IGF-1 was significantly reduced in stunted infants while alpha-1-acid glycoprotein (AGP); an acute phase protein was elevated. In high concentrations, AGP is known to inhibit thyroid stimulating hormone (TSH) signalling (Fischer *et al*., 2014). A study in Gambian children with EE reported results that were consistent with the hypothesis that microbial translocation of gut microbes and other antigen lead to chronic inflammation and systemic immune activation. The evidence of immune stimulation and inflammation was supported by elevated levels of CRP which was associated with growth faltering (Campbell *et al*., 2003). In a mouse model of bacterial translocation in an experimental intestinal obstruction model, albino rats were divided equally into groups comprising of the control, simple intestinal-obstruction and strangulated obstruction groups. CRP was one of the surrogate biomarker used for bacterial translocation. The study found the strangulated obstruction mice with the highest bacterial count in the MLNs, liver, and blood which was supported by mucosal injury score and inflammatory cell infiltrate in the intestinal wall. The results showed correspondingly high levels of plasma CRP (El-Awady *et al*., 2009). Related studies elsewhere have reported similar results (Cevikel *et al*., 2004) suggesting inflammation due to bacterial translocation which also has been suggested in EE (Korpe and Petri, 2012). In a case-control study conducted in Zambia, levels of CRP were not different between in HSS patients and healthy controls (Sinkala *et al*., 2016) suggesting that CRP may not be a good biomarker of microbial translocation.

CHAPTER THREE: METHODOLOGY

This chapter describes in detail the investigations, materials, and methods carried out in this study. It covers the recruitment of participants, sample size justification, description of sample collection and processing as well as all assays which were performed. This chapter also describes both statistical methods used for data analysis and ethical considerations.

3.1 Study Design

Unmatched case-control study with two comparison groups. The cases were individuals with EE and were compared to participants with HSS infection and healthy controls who did not have both EE and HSS infection.

3.2 Study Site

The site was chosen based on many EE studies that have been conducted for eighteen years that have demonstrated that EE is widespread (Kelly *et al*., 2004; Kelly *et al*., 2006; Louis-Auguste *et al*., 2014; Kelly *et al*., 2016). Participants with environmental enteropathy were recruited from Misisi compound. Misisi compound is an unplanned high-density settlement in the southern part of Lusaka, which is the capital city of Zambia, with an estimated population of about 90,000 people. It has poor sanitation, insufficient quality of drinking and housing compounded by HIV and AIDS, which is a huge public health problem and mortality rate in adults are high Other health

problems include malnutrition, malaria as well as cholera which is more of a seasonal disease due to inadequate clean drinking water (Central Statistics Office, 2014).

3.3 Study Population for Participants with Environmental Enteropathy

The study population were residents of Misisi compound in Lusaka, Zambia. For HSS participants they were recruited from the University Teaching Hospital as well as healthy controls those who underwent endoscopy and were found not to have EE or HSS disease.

3.3.1 Recruitment and Selection of Participants with Environmental Enteropathy

Adult males and females aged 18 years and above were recruited. The process for enrolment of participants was as follows:

Door-to-door sensitisation: The study commenced with door-to-door (house-to-house) awareness of the whole community informing community members about the study.

Participants who were willing to take part in the study were further involved in focus group discussions that included providing community members with details of the study. During the discussions, everything about the study including the aims, all procedures to be involved, and the information that was to be collected was explained. Community members were given opportunity to ask questions or raise any concerns they felt. An additional information sheet (Appendix III) was also provided for the detailed explanation of the study.

Recruitment: At this stage, participants were screened through the process of asking questions from the questionnaire (Appendix VIII) to ascertain whether they were eligible for the study. To be included in the study, participants had to sign a consent form (Appendix VI) as required by the

University of Zambia Biomedical Research Ethics Committee and clinical examination assessment was carried out on those who met the inclusion criteria. Full information about the study was and the study process was given through participant information sheet (appendix VI).

3.3.2 Inclusion Criteria for Participants with Environmental Enteropathy

The following were the criteria used to include participants in the study:

i. Adult males and females participants18 years and above

ii. No evidence of systemic disease

iii. Participants who voluntarily consented to participate in the study

3.3.3 Exclusion Criteria for Participants with Environmental Enteropathy

The following factors were used as exclusion criteria:

i. Community members included in another study
ii. Persons with helminthic infections
iii. Pregnancy
iv. Those suffering from diarrhoea at the time of recruitment or within the previous one month
v. Those on antibiotic treatment or those who have been on antibiotic treatment within 2 weeks before the study.

3.3.4 Assessment of Participants with Environmental Enteropathy

After detailed explanation was given to the participants by the gastroenterologist who performed the endoscopy procedure, also other details about sample collection, sample transportation, and laboratory procedures were explained to participants. Only individuals who met the inclusion criteria and signed the consent form were assessed at UTH in Lusaka by endoscopy to determine the EE status. EE was evaluated during endoscopy, using confocal laser endomicroscopy (CLE) followed by binocular microscopy immediately after endoscopy as well as by formal morphometry of duodenal biopsies after endoscopy. Endoscopy results showed varying degrees of abnormality entirely characteristic of environmental enteropathy. After the procedure was completed transport refund was given to the participants and no financial benefit to participants.

3.4 Recruitment and Selection of Participants with Hepatosplenic Schistosomiasis

Hepatosplenic schistosomiasis patients with oesophageal varices and/or gastric varices were recruited from clinic 5, from the endoscopy unit of the UTH in Lusaka between September 2015 and April 2016.

3.4.1 Inclusion Criteria for participants with Hepatosplenic Schistosomiasis

The criteria used to include participants in the study were concerning hepatosplenic schistosomiasis

i. Males and females aged 18 years and above
ii. Previous haematemesis
iii. Those with splenomegaly
iv. Those with oesophageal and or gastric varices
v. Those whose test results were serologically-positive for schistosomiasis
vi. Participants who consented

3.4.2 Exclusion Criteria for Participants with Hepatosplenic Schistosomiasis

The exclusion criteria were as follows:

i. Persons with alcohol dependency which was ascertained by question only

ii. Persons with cirrhosis as determined by ultrasound examination

iii. Persons who were seropositive for hepatitis B or C viral infection

3.5 Selection of Healthy Controls

Those that underwent endoscopic examination and were found to have a normal gut without EE, without HSS disease and 18 years or older were recruited as healthy controls.

3.6 Sample Size Determination

The sample size of 40 participants per group was determined with the assumption that the standard deviation of TNF-α is 27.5pg/ml and an effect size of 18pg/ml (Pastor Rojo *et al*., 2007) 95% confidence interval, 80% power and with 5% non-response rate as follows:

Table 3.1: calculation of sample size considering alpha (α) and beta (β) errors

Study characteristics	Assumptions
Type of study	An unmatched case-control (2 comparison groups)
Data sets	Observations in cases and 2 comparison groups
Variable	biomarker levels (TNF-α)

Non-response rate	5%
Standard deviation (s)	27.5
Data for alpha ($Z\alpha$)	P=0.05; 95% confidence desired (two-tailed test); Za=1.96
Data for beta ($Z\beta$)	20% beta error; 80% power desired (two tailed test); Zb=0.84
Difference to be detected (d)	18pg/ml mean difference between the environmental enteroapathy group and healthy controls

Sample size (N) $N = \frac{(Z\alpha+Z\beta)^2 \times 2 \times (s)^2}{d^2} = (1.96 + 0.84)^2 \times 2 \times (28)^2 / (18)^2$

$= 7.84 \times 2 \times 784 / 329 = \mathbf{37}$

Adjustment for expected loss to non-response rate was set at 5% =39, N = 40 participants per group x 3 groups = 120 participants total. The total number of participants needed in this study was **120**. However, they were 66 participants in the environmental enteropathy group, 86 in the hepatosplenic schistosomiasis group and 41 participants in the healthy control group. Therefore, total number of participants enrolled was **192**.

3.7 Haematological Analysis

To obtain information about the baseline characteristics of the study participants, full blood count was analysed as follows: Dipotassium ethylenediamine-tetraacetate (K2-EDTA)-containing evacuated vacutainer tubes (Becton Dickinson, New Jersey, USA) were used to draw 10ml of blood by venipuncture from all participants. The samples were transported within one hour of the collection to the Haematology Laboratory at the UTH in Lusaka and analysed on Sysmex XT 4000i automated Haematology Analyser (Sysmex Corp., Kobe, Japan). Before performing the full

blood count, three levels of internal quality control samples (low, normal, high) were run following the standard operating procedure (SOP) of the laboratory. The analyser was used to quantify red blood cells, platelets, mean pack volume, mean cell volume and haematocrit. It is capable of giving results on neutrophils, lymphocytes, monocytes, and eosinophils as percentages of the total white cell count which were stained by fluorescent dyes that bind both RNA and DNA. The instrument is also capable of determining the shape, size and density granules and nucleus of the cell by utilizing the property of side scatter (SSC) technology while fluorescence characterizes the white cell populations and scatter measurements.

3.8 Determination of CD4 Cell Count using Fluorescence-Activated Cell Sorting (FACS) Calibur

To obtain CD4 count results for those individuals who were HIV-positive, the following method was used: Venous blood samples were collected for CD4 cell count. The CD4 cell count measurements were performed on FACSCalibur flow cytometer (Beckton Dickinson, Immunocytometry System, California, USA). Before measurements, the instrument was calibrated using standardized bead kit, FACS TruCOUNT™ (BD Biosciences, California, USA). CD4-Chex Plus BC (Streck, Nebraska, USA) with controls (low and high) were run every day in the FACSCalibur system in the Virology Laboratory at the UTH in Lusaka before samples were analysed to ensure accurate and reliable results. The cell acquisition flow rate was set between 50,000 to 100, 000 lymphocyte events per tube. Determination of CD4 cell count was performed by getting 50µl of well-mixed whole blood form an EDTA tubes, which was then incubated at room temperature in the dark for 15 minutes. After incubation, 450µl of 1X lysing solution was

added, and the contents were thoroughly mixed by vortexing and incubated in the dark for another 15 minutes followed by acquiring on the instrument. The CD4 counts from whole blood were determined as cells per microliter of blood.

3.9 Determination of HIV Status

To obtain baseline HIV status of all participants, rapid determine HIV kit was used as follows: HIV test was performed using rapid HIV test kit (Alere Determine™ HIV-1/2 Ag/Ab Combo, Massachusetts, USA) from plasma (only EDTA) specimens stored at 2-8°C for not more than 7 days. Before the test, all samples and kit content were equilibrated at room temperature (18-25°C) before performing the test. The desired number of test strips were taken and marked with specimen study codes followed by removal of the protective foil cover from the test strips. Then 50µl of the specimen was applied to the sample pad using a pipette. The results were read after 15 minutes but not later than 60 minutes, and the results were entered into a table.

3.10 Quantification of Plasma Lipopolysaccharide

To answer part of objective 1, lipopolysaccharide was measured in plasma of participants as follows: The measurement of lipopolysaccharide (LPS) in plasma samples was performed by Limulus Amoebocyte Lysate Chromo (LAL), (Associates of Cape Cod Incorporated, Falmouth, USA). Reagent water (8.5ml) from the kit was used to reconstitute the standard control endotoxin (CSE) to a concentration of 1000 EU/ml followed by incubation for overnight at 2-8°C followed by a 2-fold serial dilution to make concentrations of 5.12EU/ml, 2.56EU/ml, 1.28 EU/ml, 0.64EU/ml, 0.32EU/ml, 0.16EU/ml, 0.08EU/ml and 0.04EU/ml used to draw a standard curve. All samples were diluted to a ratio of 1:1000 using reagent water provided in the kit. LAL pyrochrome reagent powder was reconstituted with 3.2 ml pyrochrome reconstitution buffer. To the endotoxin-

free ELISA plate, 50ml of the sample was added followed by addition of 50ml to all wells containing samples. After incubation for 32 minutes at 37°C, the reaction was stopped by adding 50µl of 50% acetic acid and the plate was immediately read on a microplate reader (Bio Tek EL 800, Swindon, UK) at the wavelength of 405nm. The assay was conducted in accordance with the manufacturer's instructions.

3.11 Quantification of Plasma Lipopolysaccharide-Binding Protein

To answer part of the objective 2, measurement plasma lipopolysaccharide-binding protein (LBP) was performed as follows: To measure plasma LBP a DuoSet ELISA development system (R&D Systems, Abingdon, UK) was used. A 96-well plate (R&D systems, Abingdon, UK) was first prepared by coating it with 100ml per well of diluted capture antibody in Phosphate Buffered Saline (PBS) without carrier protein then it was sealed with plate sealer and incubated overnight at room temperature. After incubation, all the wells were aspirated and washed with 400ml of wash buffer, and the process was, performed 2 times for a total of 3 washes. Buffer droplets were completely removed at each step and at the last wash any wash buffer was aspirated to dry the samples completely. The plate was blocked by adding 300ml of reagent diluent to each well then incubated for 1 hour at room temperature. Then followed by the washing of the plate as described above and was ready for sample addition. To each well 100ml of sample or standards in the reagent, diluents were added to samples followed by covering with an adhesive strip and incubated for 2 hours at room temperature. The plate wash was repeated just like during plate preparation, and 100ml of detection antibody which was diluted in reagent diluents was added to each well. A

plate was covered with a clean adhesive strip and incubated for 2 hours at room temperature in the dark. The plate was washed in the same way like during plate preparation step followed by addition of 100ml of working dilution of streptavidin-HRP to all well. The plate was incubated for about 20 minutes at room temperature in the dark. Washing of the plate was done similarly, and to each well 100ml of the substrate, the solution was added and in incubated for 20 minutes at room temperature in the dark. To each well 50ml of the stop, the solution was added, and the contents were thoroughly mixed. A microplate reader, Bio Tek EL 800, was set at 450nm to determine the optical density of each well without delay. Wavelength correction was done by subtracting the reading at 540nm from reading at 450nm to correct for optical imperfections in the plate. The assay was performed according to the manufacturer's instructions.

3.12 Quantification of Plasma Soluble CD14

To answer part of objective 2, plasma soluble CD14 was quantified as follows: A 400-fold dilution of the sample was prepared by diluting 2.5µl of the sample in 997.5ml of calibrator diluent RD5P. Reagents and working standards were also prepared according to manufacturer's instructions. 100µl of diluent assay RD1W was then added to each well, followed by 100µl of either the standards or the sample in the designated well according to the microtitre plate layout. The plate was covered and protected from direct light with an adhesive strip and left to incubate for 3 hours. After the incubation, the liquid was aspirated from the wells, and the plate washed with wash buffer four times. Wells were completely dried by blotting with the paper towel. Then 200µl of sCD14 conjugate was added to each well, after which the plate was sealed with the adhesive strip and left to incubate at room temperature for one hour. The wash step was repeated, four times after which 200 µl of substrate solution was added to each well then followed by incubation for 30 minutes in

the dark. The 50µl of stop solution was then added to each well, and the optical density of each well was determined immediately after the addition of stop solution using a plate reader, Bio Tek EL 800, set to 450 nm with the reference wavelength of 540 nm. The assay was conducted in accordance with the manufacturer's instructions.

3.13 Quantification of Human Plasma CD163

To answer part of objective 2, plasma sCD163 was quantified as follows: All reagents for soluble CD163 (R&D system, Cat No. 1630, Abingdon, United Kingdom) including samples were allowed to thaw to room temperature. To a microplate strip, 100ml of the assay diluent was added to each well followed by 50ml of standards, controls, and samples to the wells. The plates were incubated in darkness at the temperature between 18-25°C for 120 minutes. Each well was aspirated and washed four times with the buffer. After the last wash, any remaining wash buffer was removed by blotting on clean paper towel. A 200ml of the human CD163 conjugate was added to each well, and the plate was sealed and incubated in darkness for 2 hours at room temperature. Washing was repeated three times with wash buffer followed by addition of 200ml of substrate solution to all the wells and incubated for 30 minutes at room temperature away from light. After incubation, 50ml of the stop was added to each well, and the colour in the wells changed from blue to yellow. The optical density for each well was measured within 30 minutes using a microplate reader, Bio Tek EL 800, set to 450nm with the wavelength correction set at 540nm to correct for incorrect imperfections in the plate.

3.14 Measurement of Plasma C - reactive protein

To answer part of objective 2, plasma C-reactive protein was measured as follows: A 96-well Plate (R&D Systems, Abingdon, UK) was first prepared by coating it with 100ml per well of diluted capture antibody in PBS without carrier protein then it was sealed with plate sealer and incubated overnight at room temperature. After incubation, the plate was washed with 400 ml of wash buffer in each well, and the process was repeated two times for a total of three washes. The wash buffer was completely removed at each step and the last wash any wash buffer was aspirated, and the plate blotted dry on paper towel. Blocking was done by adding 300ml of reagent diluent to each well, and the plate was incubated for 60 minutes between 18-25°C after which it was washed as above. To each well 100ml of sample or standards in the reagent, diluent was added, and the plate was covered and sealed then incubated for 2 hours at room temperature. The plate wash was repeated just like during plate preparation, and 100ml of detection antibody diluted with the reagent diluent was added to each well. It was sealed and incubated for 2 hours at room temperature in the dark after which it was washed followed by addition of 100ml of working dilution of streptavidin-HRP to each well. The plates were placed in the dark away from light for 20 minutes at room temperature. The plate was washed, and to each well 100ml of the substrate, the solution was added and placed in the dark away from light for 20 minutes at room temperature. To each well 50ml of the stop, the solution was added followed by gentle tapping of the plate to ensure a thorough mix. A plate microplate reader set at 450nm was used to determine the optical density of each well without delay. Wavelength correcting was done by subtracting the reading at 540nm from reading at 450nm to correct for optical imperfections in the plate. The assay was carried-out in accordance with the manufacturer's instructions.

3.15 Determination of Schistosomiasis by Serology ELISA

To determine schistosomiasis status for the HSS participants, schistosomiasis serological test was performed as follows: The qualitative serological assay for hepatosplenic schistosomiasis (HSS) was done using a microwell ELISA (SCI- MEDX Corporation, Denville, New Jersey, USA). The assay detects plasma IgG against schistosomal species but does not differentiate schistosomal species. The 96-well plates were used according to the numbers needed regarding controls and samples. To the appropriate wells, 100µl of negative controls, 100µl of positive and 100µl of samples which were diluted (1:40) test samples were added to the remaining. The negative and positive controls were supplied as pre-diluted. The plates were incubated at room temperature (15 - 25°C) for 10 minutes. The contents of the plate were aspirated and washed 3 times using wash buffer. To every well, 2 drops of enzyme conjugate was added followed by incubation at room temperature for 10 minutes. The contents were aspirated and washed 3 times using wash buffer. To every well, 2 drops of chromogen was added and incubated at room temperature for 5 minutes then followed by addition of 2 drops of stop solution. The ELISA reader (Bio Tek EL 800, Swindon, UK) was zero on air, and the plates were read at 490nm. The cut-off for positive samples was equal or greater than the optical density (OD) value of 0.2. The assay was performed in accordance with the manufacturer's instructions.

3.16 Extraction of DNA from Whole Blood

To answer part of objective 1, DNA was extracted from whole blood. One of the major determinants of the sensitivity of PCR bacterial DNA detection is the DNA extraction. Several methods Wizard SV genomic DNA purification system (Promega, Madison, Wisconsin, USA), 5 U lysozyme (Ready Lyse, Epicenter, Madison, Wisconsin USA) and QIAmp DNA blood mini kit (QIAGEN, Basel, Switzerland) were compared for their suitability for blood DNA extraction under the prevailing conditions. QIAmp DNA blood mini kit was found to be superior to the other

methods for extraction DNA for 16S rRNA gene amplification especially from Gram-negative bacteria (Zucol *et al*., 2006).

Plasma samples were equilibrated at room temperature (15 – 25°C) after which 20µl of QIAGEN protease enzyme was added to each 200µl of plasma sample in 1.5ml microfuge tubes. Samples which were less than 200µl were adjusted by adding appropriate volume of PBS and all samples were properly mixed after adding the enzyme. A volume of 200µl of buffer AL (containing 28µg/ml of carrier RNA) was added followed by vortexing for 15 seconds to ensure efficient lysis. The samples were incubated at 56°C for 10 minutes after which they were centrifuged to remove drops from inside the lid followed by addition of 250µl of absolute ethanol and thorough mixing for 15 seconds. The mixtures were transferred to the 2ml QIAamp spin column and centrifuged at 8000 rpm for 1 minute, and the filtrate was discarded. The QIAamp spin columns were transferred to clean collection tubes, and each spin column was closed to avoid aerosol formation during centrifugation. Centrifugation did the cleaning of the samples was at higher speed until the QIAamp spin column was empty. Then 500 µl buffer AW1 were carefully added followed by centrifugation at 8,000 rpm for 1 minute. Spin columns were after that transferred to clean 2ml collection tubes and 500 µl buffer AW2 was added without wetting the rim. The caps were closed, and the columns were again centrifuged at 8,000 rpm for 1 minute. Spin columns were moved to a clean 2ml collection tubes and, 500µl of absolute alcohol was added followed by centrifugation at 8,000 rpm for 1 minute. QIAamp columns were transferred to clean 2 ml collection tubes and centrifuged at 14,000 rpm to dry them. The columns were transferred to a clean 1.5ml microcentrifuge tube and 200µl buffer AVE was added and they were incubated at 56ºC for 5 minutes to increase DNA yield. DNA was eluted by centrifuging the columns at 14,000 rpm for 1 minute. The extraction was performed according to manufacturer’s instructions. The concentration

and purity of DNA was spectrophotometrically determined using Nanodrop spectrophotometer 2000c (Thermo Scientific, Massachusetts, USA). DNA was kept at -20ºC until needed.

3.17 Real-time Quantitative Polymerase Chain Reaction

Real-time quantitative PCR (q-PCR) was carried-out using broad range primers for 16S rRNA as listed in Table 3.2. All primers were reconstituted with sterile water to final stock concentrations of 1mg/ml while the working primer concentrations were 50 nanograms. Real-time quantitative polymerase chain reaction mixes were prepared. Quantitative PCR detection was performed using QuantiTect SYBR® Green PCR kit from Qiagen following the manufacturer's instructions. The qPCR was performed on a Rotor gene 6000 real time quantitative PCR machine (Rotor-gene, North West, Australia). The reaction temperature profile consisted of an initial denaturing at 95C for 15 minutes, cycle denaturation at 95C for 15 seconds, annealing was at 60C for 30 seconds and extension at 72C for 45 seconds. Real-time quantitative PCR was carried-out using broad range primers for 16S rRNA, and details of these primers are listed in Table 3.2. The Primers were reconstituted with sterile water, and the forward primer 16S F519 (5'-CAGCAGCCGCGGTAATAC-3') with the total of 249.8µg, 249.8µl of sterile water was added making a stock concentration of 1mg/ml. The reverse primer 16S R785 (5'-TGGACTACCAGGGTATCTAATCC-3') with the total of 345.2µg, 345.2µl of sterile water was added making a stock concentration of 1mg/ml. The primers were both diluted to 50ng/ml (20-fold) to get working concentration. The addition of 10µl of each primer (forward and reverse) from the stock was added to 180µl of sterile water to make 200µl. Real-time PCR set-up was performed as shown below (Table 3.2)

Table 3.2: Real-time Quantitative PCR set up

Components	**Reaction volume (20µl/tube)**	**Example – 2x QuantiTect SYBR Green mix for 50 tubes**	
2x QuantiTect SYBR green mix	10µl	X 50	500µl
Primers (forward + reverse)	0.4 µl	X 50	20µl
Genomic DNA (dispensed separately)	5µl	-	Added separately
Water	4.6µl	X 50	230µl
-	-	15µl of reaction Mix was dispensed to individual tubes followed by addition of 5µl of temperate DNA	

3.17.1 Determination of Specificity and Sensitivity of the 16S Real-time Quantitative Polymerase Chain Reaction Assay

Determination of the specificity of the 16S rRNA gene primers was done using a known quantified (*E.coli* K12 strain). A 10-fold serial dilution of a quantified plasmid was used to establish the standard curve which was used for absolute quantification starting with 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 16S rRNA copy number per microliter (Figure 3.1A) and Non-Template Control (with each dilution tested in triplicate). After PCR amplification had been done, the sensitivity of PCR and the standard curve was generated. The melting curves were generated and showed sharp peaks at the expected melting temperature (85.4°C ± 1.3) indicating specific detection of 16s rRNA gene product (Figure 3.1B), Simple linear regression of the Ct values from Standard curve plotted against log of the initial 16S rRNA gene copy number (Figure 3.1B) and quantitative PCR descriptors (3.1C).

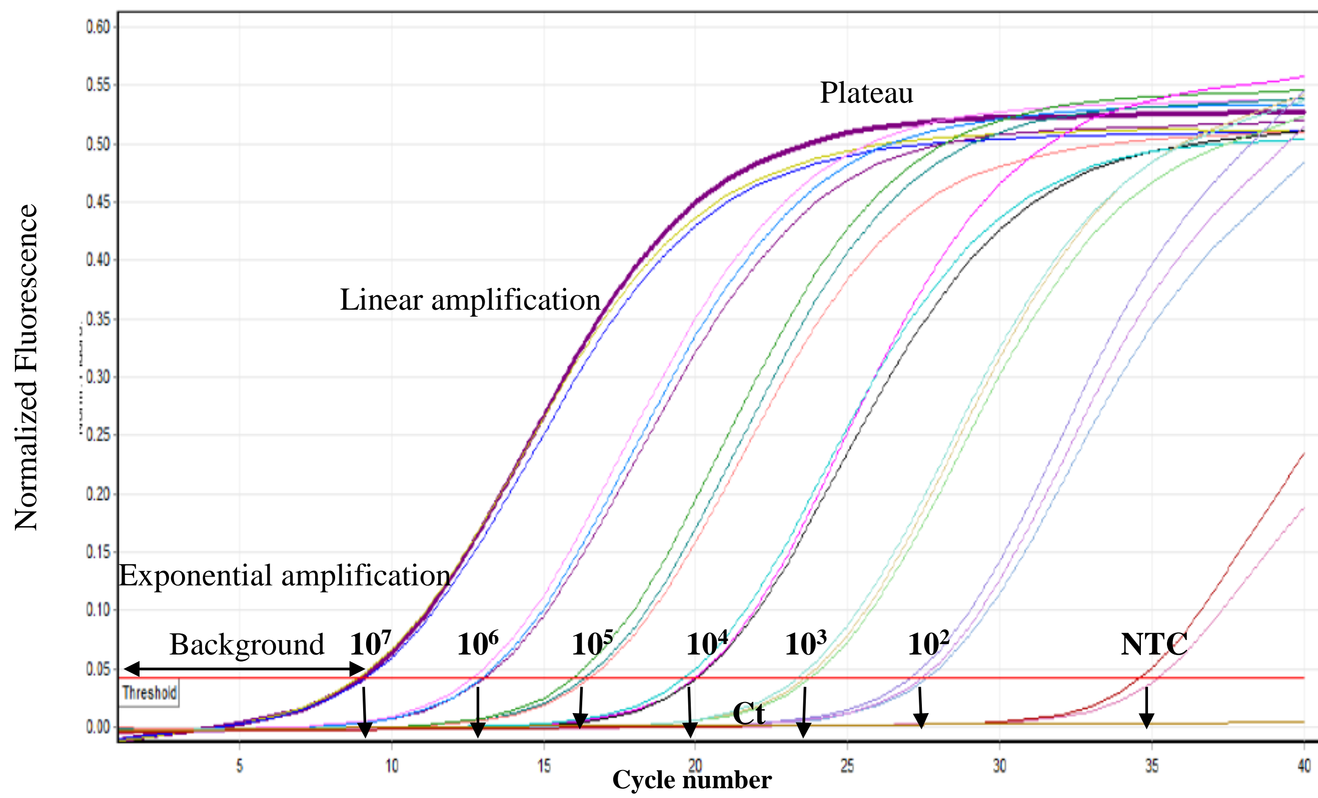
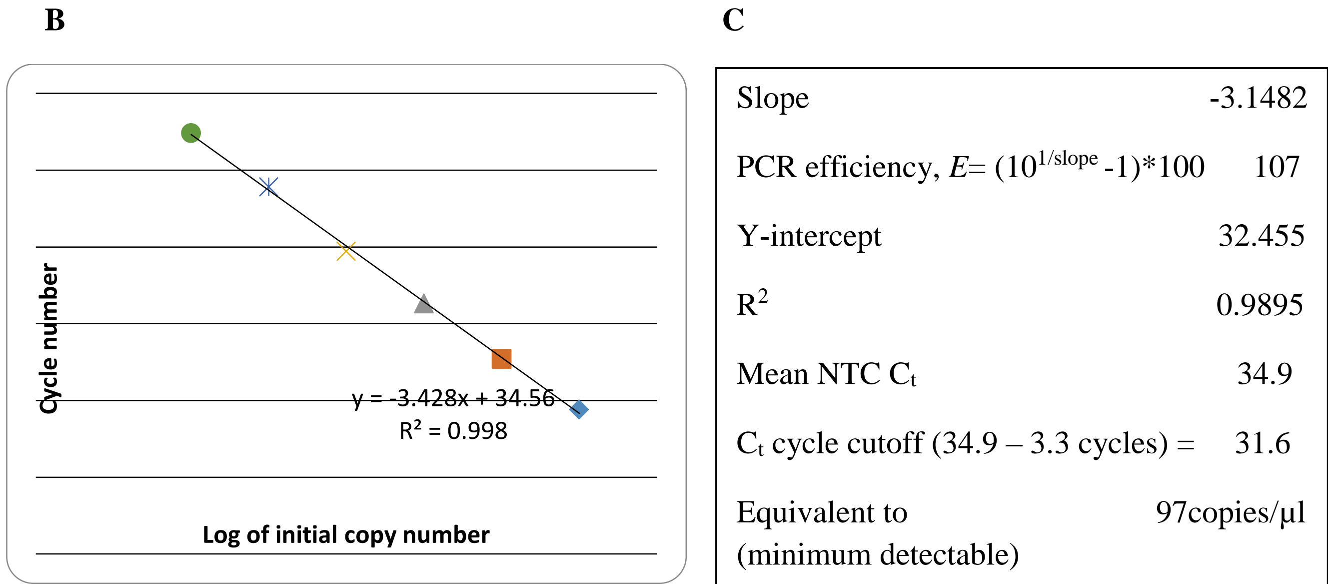

Normalized Fluorescence
Plateau
Linear amplification
Exponential amplification
Background
Threshold
10^7
10^6
10^5
10^4
10^3
10^2
NTC
Ct
Cycle number
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5
10
15
20
25
30
35
40

Figure 3.1A: Quantitative PCR amplification from quantified plasmid with known insert concentrations of template DNA used to construct standard curves for quantification of unknown blood samples.

B **C**



Slope	-3.1482
PCR efficiency, E= $(10^{1/slope}-1)*100$	107
Y-intercept	32.455
R^2	0.9895
Mean NTC C_t	34.9
C_t cycle cutoff (34.9 – 3.3 cycles) =	31.6
Equivalent to (minimum detectable)	97copies/µl

Figure 3.1B and C: Simple linear regression of the Ct values from Standard curve (above) verses log of the initial rRNA gene copy number (b). Quantitative PCR descriptors (c).

3.17.2 Melting Curve Analysis for Confirmation of 16S rRNA genes

Melting curve analysis was performed by increasing the temperature of the PCR product was over a gradient, and the double-stranded template was denatured resulting in the SYBR green dissociate from the product and matching fall in fluorescence. At every point fluorescence and temperature intensities were measured and confirmed that targeted template was present, 50% of the double-stranded template at a temperature of 83.4ºC was denatured. A melting curve analysis was carried out over a period of 20 minutes by first cooling the reaction to 60°C then heating to 99°C. Melting curve for each sample was quantified constantly during the heating process and the SYBR Green I fluorescence was plotted against temperature. The peak point in the melting curve analysis was

deemed to be the melting temperature (Tm) after plotting the negative offshoot of the fluorescence aligned with temperature (-dF/dF vs. T) as shown below (Figure 3.2).

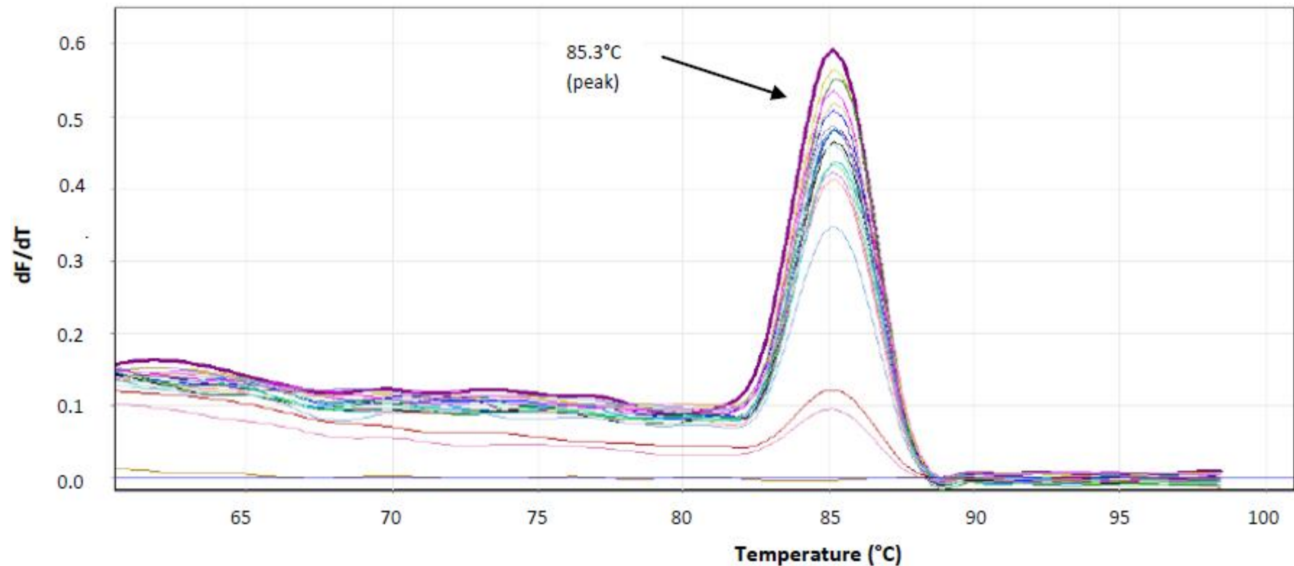


Figure 3.2: Melting curve analysis obtained after serial dilution of quantified plasmid used as standard after the quantitative PCR run.

3.18 RAW-Blue Macrophage Cell Culture

To answer part of objective 1, The Raw-Blue macrophage cells were cultured as follows: At the beginning, all external and internal surfaces of the cell culture biosafety level 2 cabinet were surface-sterilised with 70% ethanol to prevent contaminations. RAW-Blue macrophage cells were cultured in Dulbecco´s modified Eagle medium (Sigma-Aldrich, London, UK). The medium was supplemented with 4.5g/L glucose and 10% (v/v) heat-inactivated (at 56°C for 30 minutes) fetal bovine serum (FBS) (Invitrogen, San Diego, USA,) and L-glutamine to the final concentration of 2mM. The antibiotics normacin (final concentration of 100μg/ml) together with penicillin-streptomycin (final concentration 50U/ml-50μg/ml) were added. Furthermore, 0.2% (v/v)

antibiotic-antimycotic solution (AAS) (Invitrogen) and 200 µg/ml Zeocin (InvivoGen) were added to prevent bacterial and fungal contaminations of the cell culture.

All experiments involving cell culturing were performed in a Level 2 cabinet. Stability and performance of the cell cultures were ensured by preparation of stocks initially then additional stocks were kept for use in subsequent experiments. Cells in a vial were thawed rapidly by gentle agitation in a water bath at 37°C taking all precautions to avoid contamination. The vial was taken from the water bath as soon as the contents were thawed and decontaminated by spraying with 70% ethanol under strict aseptic conditions. Cells were transferred to a 15ml Corning tube containing pre-warmed (at 37°C) growth medium. The tube was centrifuged at 1200rpm for 5 minutes and, the supernatant containing cryoprotective agent was discarded and, the cells were re-suspended in 1ml of growth medium. They were then transferred to a T-25 culture flask containing 5 ml of growth medium and placed in a culture incubator at 37°C in 5% carbon dioxide under humid conditions.

To keep stocks of cells, they were re-suspended at a density of 3 – 5 x 10^6 cells/ml in freezing medium and aliquoted in 1ml cryogenic vials. The vials were stored at -80°C overnight and later transferred to liquid nitrogen for long-term storage. Growth medium was renewed twice a week and cells were passaged when 70 – 80% confluence was reached and, the cells were never allowed to reach 100% confluence.

3.18.1 Cell Counting and Viability Method

The number of cells yield and viability after culture was performed using trypan blue solution as it stains dead cells blue. Live cells can select compounds that pass through the membrane and dead ones do not, the dye enters the dead cells but excludes live ones. Any cells stained with trypan blue

solution or faint looking in any of the four big squares were considered dead. Counting of viable cells was done using the following formula:

Percentage of live cells = live cell count / the live and dead cell count multiply by 100. A hemocytometer (Sigma-Aldrich, Steinhein, Germany) was cleaned and surface-sterilised with 70% ethanol and 100µl of the cells were mixed gently with 100µl of 0.4% trypan blue solution (Sigma-Aldrich, Steinheim, Germany) while avoiding cell lysis. The 1:2 trypan dilution was added to 10µl of cell mixture and applied to the hemocytometer filling the chamber using a 100 µl Eppendorf pipette. Cells were counted (using x10 objective) in the four squares (shown in blue colour) and only live cells (unstained by trypan blue solution) and within the micrometer squares and positioned on the right hand or bottom boundary line was counted to avoid counting same cells twice. After counting all 4 big squares (in blue), the average was calculated, and the hemocytometer designed with the big squares and the number of cells counted is equivalent to x 10^4/ml. The hemocytometer indicating the 4 corner squares which were used for counting cells is shown (Figure 3.3).

Calculation of the total number of cells, the following formula was used:

$$\text{Total cells/ml} = \frac{\text{All cells counted x dilution factor}}{\text{Number of squares}} \text{ x 10,000 cells/ml}$$

$$\frac{\text{Total cells x 2 (dilution factor) x 10,000 cell/ml}}{\text{4 squares}} = \text{number of cells x } 10^4 \text{ /ml}$$

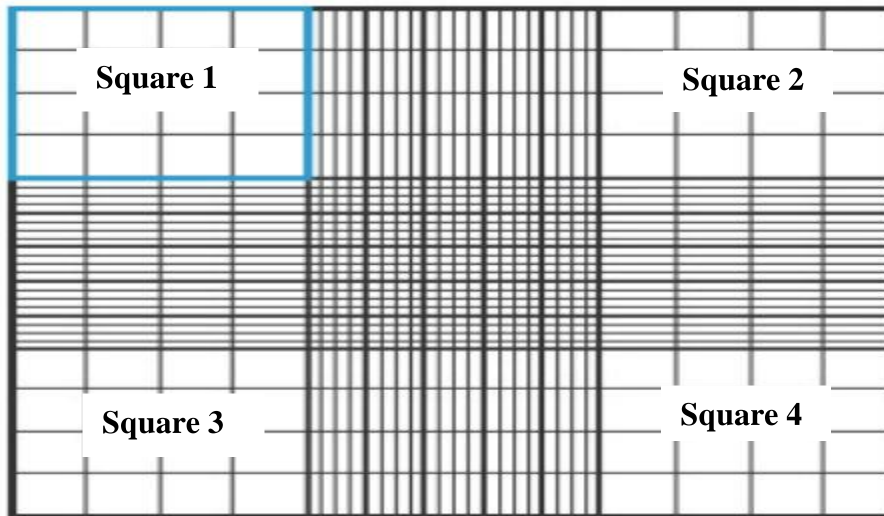


Figure 3.3: Hemocytometer indicating the 4 corner squares which were used for counting cells.

3.18.2 QUANTI-Blue™ Assay

To answer part of objective 1, The QUANTI-Blue assay was used to detect the activation of NF-κB activation after TLRs were activated by pathogen-associated molecular patterns (PAMPs) in plasma. From a T-25 flask with cell growth which had reached the 70 - 80% confluency, the medium was removed by aspiration with a disposable pipette. Cells were washed twice with phosphate buffered saline (PBS). RAW-Blue cells were detached using a cell scraper and re-suspended in test medium containing 10% (v/v) heat-inactivated FBS (heated for 30 minutes at 56°C) and a cell suspension of ~550, 000 cells/ml was prepared. Then 20µl of plasma samples were added to each well of a flat-bottom 96-well culture plate including LPS (*Salmonella enterica*, serotype typhimurium [0, 0.1, 10, 100 and 1000ng/ml]) as positive controls, two negative controls (medium only and endotoxin-free water) followed by addition of 180µl of cell suspension (~100,000 cells) per well. The cell suspension was frequently homogenised to ensure reliable and

reproducible results and the plate was incubated in at 37°C in a CO_2 incubator for 24 hours in the humid atmosphere.

QUANTI-Blue^{TM} (InvivoGen, San Diego, USA) reagent was prepared by placing all the contents of a pouch into a 250ml sterile flask followed by addition of 100ml endotoxin-free water. The flask with its content was swirled gently and warmed to 37°C for 30 minutes. Complete dissolution of the QUANTI-Blue^{TM} powder was ensured by incubation overnight at 4°C, and the completely dissolved reagent was filter-sterilized on a 0.2µm membrane.

3.18.3 Detection of Toll-like Receptor Ligands from Cell Culture Supernatant

A 150ml aliquot of warm (37°C) QUANTI-Blue^{TM} reagent was transferred to each well of a flat-bottom 96-well culture plate followed by addition of 50µl of cell culture supernatant. Different concentrations of LPS as positive controls and negative controls (endotoxin-free water) were included in the incubation and culture plate was incubated at 37°C for 1 hour. SEAP activity was determined by reading the optical density (OD) at 630nm with a microplate reader. The deep blue colour indicate positive for TLRLs and the purple indicaticate negative reaction (Figure 3.4). The assay was conducted in accordance with the manufacturer's instructions.

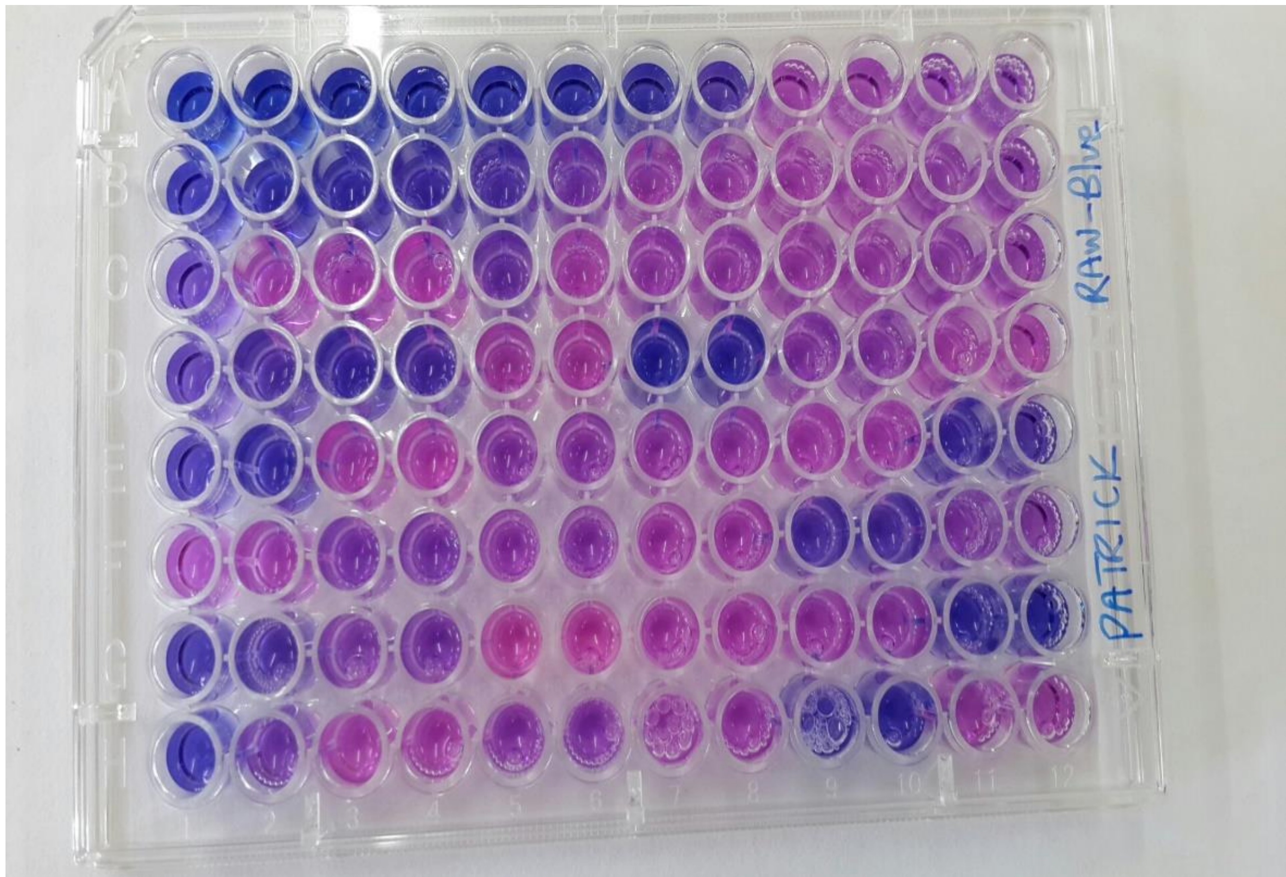


Figure 3.4: Shows QUANTI-Blue assay. The deep blue colour indicate positive for TLRLs and the purple indicaticate negative reaction.

3.19 BD^{TM} Cytometric Beads Array Mouse Th1/Th2/Th17 Cytokine Kit for the Determination of Cytokines

3.19.1 Preparation of Mouse Th1/Th2/Th17 Cytokine Standards

To answer part of objective 3, cytokines were measures in cell culture supernatant. The mouse Th1/Th2/Th17 cytokine standards (BD Biosciences, Oxford, England) were reconstituted immediately and serially diluted followed by mixing with the capture beads and the phycoerythrin (PE) detection reagent. The serial dilution procedure started with the top standard by transferring the content from the vial to a 15ml DB^{TM} conical tube polypropylene tube which was labelled top

standard. The top standard was reconstituted by using 2ml assay diluents, gently mixed by using a pipette and incubated for 15minutes at room temperature for complete equilibrium. Eight more 12 x 75-mm tubes were labelled 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 and 300ml of assay diluents was added to each tube. Serially addition of 300µl of the top standard was added to the 1:2 tube and mixed thoroughly by using a pipette followed by addition to the 1:4 tube until to the 1:256 tube. Then another tube was prepared to contain assay diluents only which serve as a 0 pg/ml negative control as shown below (Figure 3.5).

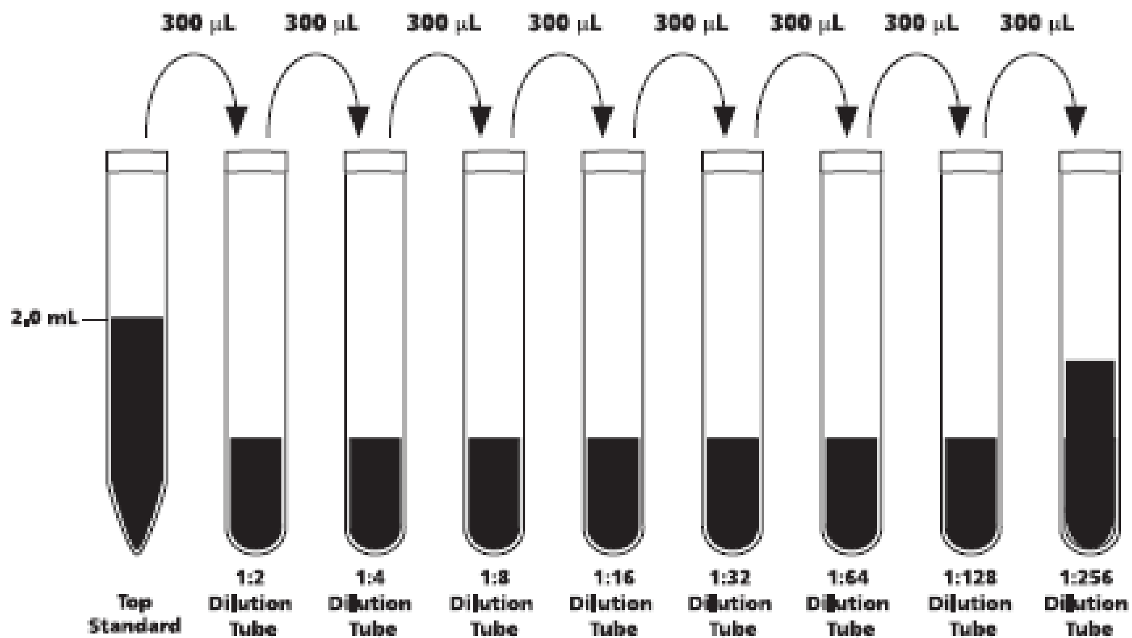


Figure 3.5: A 2-fold serial dilution preparation of cytometric beads assay standards

3.19.2 Mixing of Mouse Th1/Th2/Th17 Cytokine Capture Beads

After determining the number of assay tubes required (9 cytokine standards, 1 negative control and unknown samples), all capture beads were vigorously vortexed for 5 minutes to re-suspended them. To each assay tube, a 10µl aliquot of each capture bead was added to one single tube labelled "mixed capture beads" followed by a vigorous vortex to mix the beads adequately. The capture

beads were treated with the enhancement buffer by centrifugation of the mixed capture beads at 200g for 5 minutes, and then carefully the supernatant was aspirated and discarded. An equal volume of the enhancement buffer was used to re-suspend the mixed capture beads pellet to the one which was removed and discarded. The capture beads were then incubated protected from light for 30 minutes at room temperature after which the mixed capture beads were transferred to assay tubes.

3.19.3 Performing Mouse Th1/Th2/Th17 Cytometric Beads Assay

All mixed capture beads were vortex and 50µl were added to all assay tubes followed by addition of 50µl of the Th1/Th2/Th17 cytokine standard dilutions to the control tubes. Also, 50µl of each unknown cell culture supernatant samples were added to appropriately labeled tubes followed by another addition of mouse Th1/Th2/Th17 PE detection reagent to all assay tubes. Incubation of the tube was for 3 hours at room temperature protected from sunlight. After incubation, to each assay tube, 1 ml of wash buffer was added and centrifuged at 200g for 5 minutes followed by carefully aspirating for the supernatant from each tube and discarded. To re-suspend the bead pellet 300ml of the wash buffer was added to each assay tube and the samples were acquired on the flow cytometry (FACSVerse). The dilutions of the standards are shown below (Table 3.3).

Table 3.3: Th1/Th2/Th17 cytokine standard dilutions

Tube	Concentration (pg/ml)	Cytokine Standard dilution
1	0 (negative control)	no standard dilution (Assay diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16
7	625	1:8
8	1,250	1:4
9	2,500	1:2
10	5,000	Top standard

3.19.4 FACSVerse Instrument Setting

For flow cytometry, BD FACSVerse was used, and some procedure was followed before acquiring samples.

3.19.5 FACSVerse - Daily Clean

Performed every day before and after running samples even before shutting down the instrument. From the menu bar, the option of cytometer then daily clean was highlighted and clicked. A choice of daily clean was given and selected. Bleach (10%) was prepared by (10ml bleach and 90ml distilled water) was put into 12 x 75mm tube. The tube containing 10% bleach was placed on the aspiration port and then to continue option was selected, and immediately aspiration started. After aspiration, the tube was removed and the window closed. A tube containing distilled water was

placed in the manual port immediately and to continue option was selected. Then was followed by purging sheath filter twice with distilled water by pressing cytometer followed by fluidics then purge sheath filter. The flow cell was then drained twice using distilled water by clicking cytometer then fluidics followed drain and fill flow cell. After the all the above functions were performed, FACSVerse was ready for performance quality control run.

3.19.6 Performance Quality Control for FACSVerse – Daily

The setup and quality control (QC) were performed every time before samples were run on the instrument. When new reagents box for cytometer setup beads (CS and T) beads is received, to get the lot number, the BD website, bdbiosciences.com was visited to get access to CS and T research beads. The appropriate bead lot file corresponding to a current lot of CS and T research beads was downloaded and imported, then the number was entered into the instrument. The CS and T research beads were thoroughly mixed using the vortex to make sure that a homogeneous mixture was achieved. The 12 x 75 mm was taken and labelled. After labelling, 0.5ml of sheath fluid was dispensed into the tube. Then two drops of the beads were put into the labelled tube and mixed with the sheath fluid thoroughly. Then once again the tube was vortexed just before use. The CS and T bead lot number was selected. The performance quality control option was selected and started. After the performance QC passed and had passed then, the instrument was ready for sample acquisition.

3.19.7 Characterization Quality Control for FACSVerse - Monthly

The monthly cleaning was as the daily clean except the 1.0ml sheath fluid was used and not 0.5ml. Four drops of the CS and T research beads were added and not two. On the setup and QC

option, the setup and QC tasks were selected. The CS and T bead lot was selected. Using the characterization QC icon, the QC was started.

3.19.8 Flow Cytometer Adjustment

The instrument was set to acquisition mode after appropriate instrument setup temperate and forward scatter area (FSC-A) and side-scatter area (SSC) was set to log mode. The instrument side-scatter area (SSC-A) SC PMT voltage settings and threshold were optimized to acquire 10, 000 cells by addition of 50ul of cytometer setup beads (CS & T) beads to 450 of wash buffer. BD FACSverse and the threshold were set to FSC at 650, and all compensation values were set to 0.0%. The beads were run in a setup mode then paused and restarted acquisition frequently during the configuration procedure after settings adjustment to reset detected values. To get a single bead population gate R1 was adjusted. FL3 or FL4 PMT voltage was changed, so that bead population intensity was as close as possible to 5000 as the median of the top FL3 or FL4.

3.19.9 Acquiring the Samples on the Flow Cytometer

To answer part of objective 3, Samples were acquired on the BD FACSVerse flow cytometer (BD Biosciences, San Jose, USA) beginning with standards from lowest (0 pg/ml) to highest (Top standard) concentration then followed by test samples. The acquisition and storage window resolution was set at 1024 pixels and to reduce the chances of missing weakly stained samples; a medium flow rate was set to collect 2100 events. It was ensured that the sample file contained thoroughly 300 events per capture bead (BD Bioscience, 2015). The number of events to be collected was set to "all events" and it was saved to ensure that no actual bead events were lost to incorrect gating. Analysis of results was done using FCAP Array software (BD Biosciences, San

Jose, USA). Sample intensities that were not falling within the limits of the standard curve were extrapolated by applying the 4-parameter curve fit option as recommended by BD Biosciences. According to the kit, the theoretical of detection limits for the cytokines were IL-2: 0.1 pg/ml, IL-4: 0.03 pg/ml, IL-6: 1.6 pg/ml, IL-10: 16.8 pg/ml, TNF-α: 0.9 pg/ml, IFN-γ: 0.5 pg/ml and IL-17A: 0.8 pg/ml.

3.20 Data Analysis of Cytokines using FCAP Array Software

FCAP array software v3.0 software (BD Biosciences, San Jose, USA) was used to analyse the data. It analysed multiple analytes concentrations of data from the CBA kit quantitatively based on the concentration standards. The software uses FCS data files to which the analytes have been assigned, and the using the detector antibody for each analyte are determined using median fluorescence intensity (MFI). Sample concentrations were determined by calculations from the standard curve. BD CBA offers a faster and simple way for the acquisition of BD CBA experiments and later analysis with the use of FCAP Array software when used together with BD FACS Verse analyser and BD FACS Suite software. The cytokines analysed were TNF-α, IL-6, IL-10, IL-4, IL-2, IFN-γ and IL-17A.

3.21 Data Analysis

For the comparison of baseline characteristics among EE, HSS patients, and healthy controls, the chi-squared test was used for categorical variables and the Kruskal-Wallis test followed by Dunn's post hoc test for continuous variables. Normality test of data showed that the data was not normally distributed using Shapiro-Wilk test. Biomarkers of host immune response to microbial translocation were also analysis using Kruskal-Wallis. Understanding of the relationships among biomarkers and between biomarkers and cytokines, Spearman's rank correlation was used. A

correlation matrix was done between direct biomarkers of microbial translocation and biomarkers of host immune response to microbial translocation. In this kind of analysis there is an element of multiple testing so Bonferroni was used to adjust but this test over adjusting. This was followed by Benjamini-Hocberg for discovery of false positive rate. After adjustment for baseline characteristics, taking direct biomarkers as independent variables and cytokines as dependent variables, multivariate multiple regression was conducted with the probability of removal in the final model set at $p<0.20$ (20%) and interpreted using regression coefficients and confidence intervals. After running regression models, Breusch-Pagan test was run to test for heteroskedasticity for all independent variables using the (***hettes***t command in STATA). Model fit was tested using Aikaike Information Criteria and Bayesian Information Criteria. All statistical analyses of data were done using GraphPad Prism version 6.01 (GraphPad Software Inc., La Jolla, CA, USA) and STATA version 13 (Stata Corp, College Station, Texas, USA). For all statistical tests, a p value of <0.05 was considered statistically significant.

3.22 Ethical Considerations

Permission and ethics approval were obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC), and informed consent from all participants was obtained and confidentiality was ensured. There were no names used on participants' records only codes were used. Whether the participants declined or not to participate in the study, standard care was given to the participants. There was no coercion of any kind for anyone to participant in the study. The study was explained to the participants, and both oral and written consent was obtained from the participants before participation in the study. The endoscopy procedure was explained to the participants by the gastroenterologist who performed the procedure. The procedure and sedation drugs that were used the risks and benefits were explained to the participants. However, incidence

of risk such as over sedation and bleeding during endoscopy are very rare as low as 1 in 10,000. But even if these risks were due to happen, they rarely become fatal and in fact physician were always around in case of such eventualities to ensure safety of the participants.

3.23 Limitations of the Study

The present study was limited as the recruitment of healthy controls was from the hospital that came to seek medical care. It is possible that though endoscopy results showed that they were normal and they did not have HSS disease, they could have had other conditions that may predispose them to microbial translocation that the study did not detect at enrolment stage. However, endoscopy on healthy normal individuals may also raise ethical issues so this was the best possible participants that were enrolled in the study. The study included a limited number of biomarkers of host response to MT. However, these biomarkers are the commonly reported in literature in the MT studies. Additional biomarkers such as interleukin-1 (IL-1) and interleukin-12 (IL-12) could be considered in future studies to elucidate the EE and HSS conditions. Other biomarkers such as Intestinal Fatty Acid Binding Protein (I-FABP) that may be used to evaluate gut damage, especially in individuals with EE. The study cannot establish the causal relationship between direct biomarkers and immune activation due to the nature of study design.

CHAPTER FOUR: RESULTS

4.1 Baseline Characteristics of Study Participants

In the EE group, males were 22 (32.8%). In the HSS participants males were 34 (39.5%). In the healthy controls male were 20 (48.7%). The sex ratios for all three groups of study participants were not statistically significant (p=0.41). The age of HSS participants had a median of 40 (IQR, 30 – 51) years and were older compared to the EE participants with a median age of 29 (IQR, 24 – 43) years or healthy controls with a median of 32 (IQR, 25 – 38) years; p=0.001). The body mass index (BMI) for EE participants had a median of 22.9 (IQR, 20.5 - 27.6), HSS participants had a median of 22.7 (IQR, 21.4 - 26.9) and healthy controls had a median of 23.6 (IQR, 21.2 - 28.3). BMI was statistically different across the groups; p = 0.22. In the EE participants those with secondary education level and higher were 25 (39%), HSS participants were 33 (38%) and healthy controls were 15 (42%) and there was no statistical significant difference among the groups; p = 0.80. HSS participants were more likely to have lower hemoglobin with a median of 9.7 (IQR, 6.4 - 11.5) g/dL, white cell count with a median of 2.5 (IQR, 2 - 4.1) $x10^9$/L and platelets with median of 121 (IQR, 87 - 137) x 10^9/L; p<0.0001 (Table 4.1). Participants with EE had a substantial prevalence of HIV (22%); p<0.001) compared to HSS participants or healthy controls of which none had the infection (Table 4.1).

Table 4.1: Baseline Characteristics of Study Participants

Variable	EE (n = 67)	HSS (n = 86)	Controls (n = 41)	P value
Sex (M:F)*	22:45	34:52	20:21	0.41
Age	29 (24 - 43)	40 (30 - 51)	32 (25 - 38)	0.01
Education, secondary or more (n, %)*	25 (39)	33 (38)	15 (42)	0.80
BMI (kg/m^2)	22.9 (20.5 - 27.6)	22.7 (21.4 - 26.9)	23.6 (21.2- 28.3)	0.22
Hb (g/dl)	13.3 (12.5 - 14.9)	9.7 (6.4 - 11.5)	13.6 (11.2 -18.3)	<0.001
Platelet count (x10^9/l)	232 (200 - 280)	121 (87 - 137)	189 (143 - 230)	<0.001
White cell count (x10^9/l)	4.2 (2.8 - 4.9)	2.5 (2 - 4.1)	4 (4 - 7)	<0.001
HIV sero-positive	14 (22)	0(0)	0(0)	<0.001
CD4 count (cells/µl)	516 (350 - 694)	_	_	_

BMI = body mass index; EE = environmental enteropathy; Hb = haemoglobin; HIV = human immunodeficiency virus; HSS = hepatosplenic schistosomiasis; F = female; M = male. *chi square was used; percentages are in parentheses. For continuous variables median (IQR) are shown.

4.2 Endoscopy Results

To check for enteropathy for all the three groups of participants, endoscopy was performed. The EE participants had various degrees of enteropathy. In the HSS participants, 60 (69%) had oesophageal varices, 18 (24%) had gastropathy and 8 (16%) had gastric varices. All healthy controls had no EE, HSS, oesophageal varices and gastric varices or gastropathy.

4.3 Comparison of Direct Biomarkers of Microbial Translocation

Objective 1: To compare direct biomarkers of microbial translocation in participants with environmental enteropathy, hepatoslpenic schistosomiasis and healthy controls.

4.3.1 Comparison of Lipopolysaccharides among the EE, HSS and Healthy Controls

The LPS levels were significantly higher in the EE participants, median was 379 (IQR, 82.7 – 870) EU/ml compared to HSS participants 213 (IQR, 77.2 – 358); p=0.03 or healthy controls 202 (IQR, 43.2 – 251); p=0.01. There was no significant difference between HSS participants and healthy controls (p=0.08) as shown in Figure 4.1. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used to compare all pairs. The results are shown with significance where applicable ($p < 0.05$).

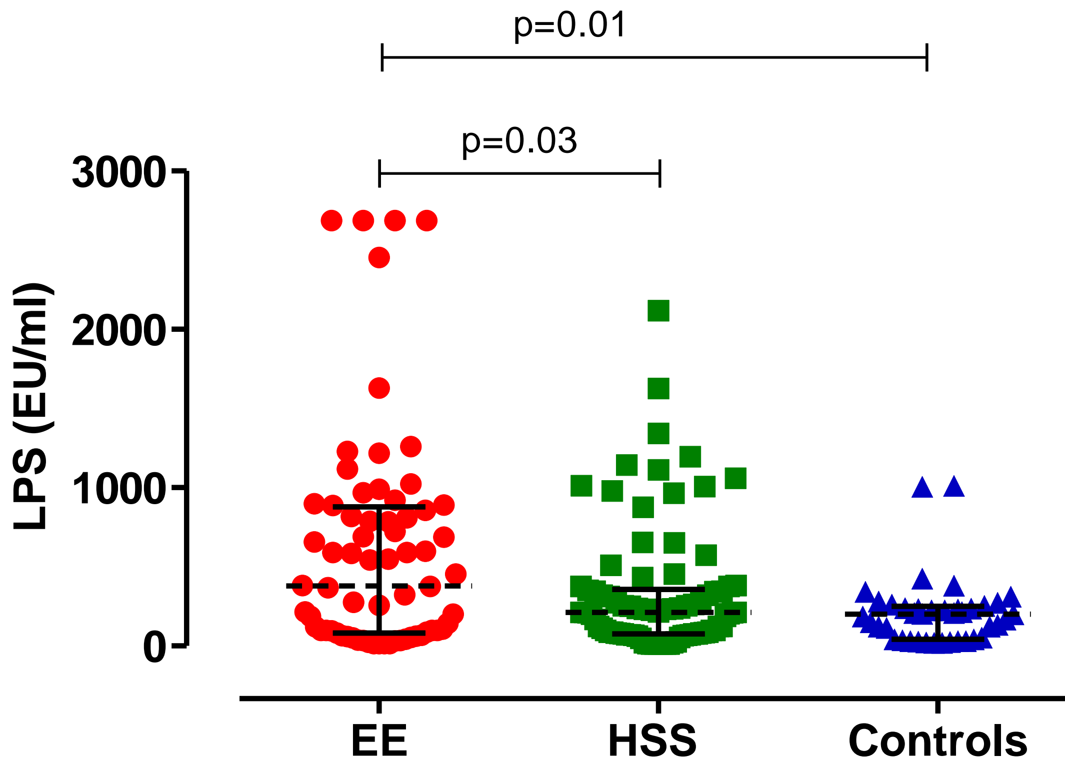


Figure 4.1: Comparison of plasma lipopolysaccharide among EE, HSS and healthy controls. The horizontal broken lines within the dot plots are medians.

4.3.2 Comparison of 16S rRNA gene copies among the EE, HSS and Healthy Controls

The 16S rRNA copy number was significantly higher in participants with EE, median was 2651 (IQR, 529 – 8779) copies/µl compared to HSS participants 387 (IQR, 165 – 1990); p<0.001 or healthy controls 193 (IQR, 132 – 453); p<0.001. There was also significant difference between HSS participants and healthy controls (p=0.003) as shown in Figure 4.2. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used compare all for pairs. The results are shown with significance where applicable (p< 0.05).

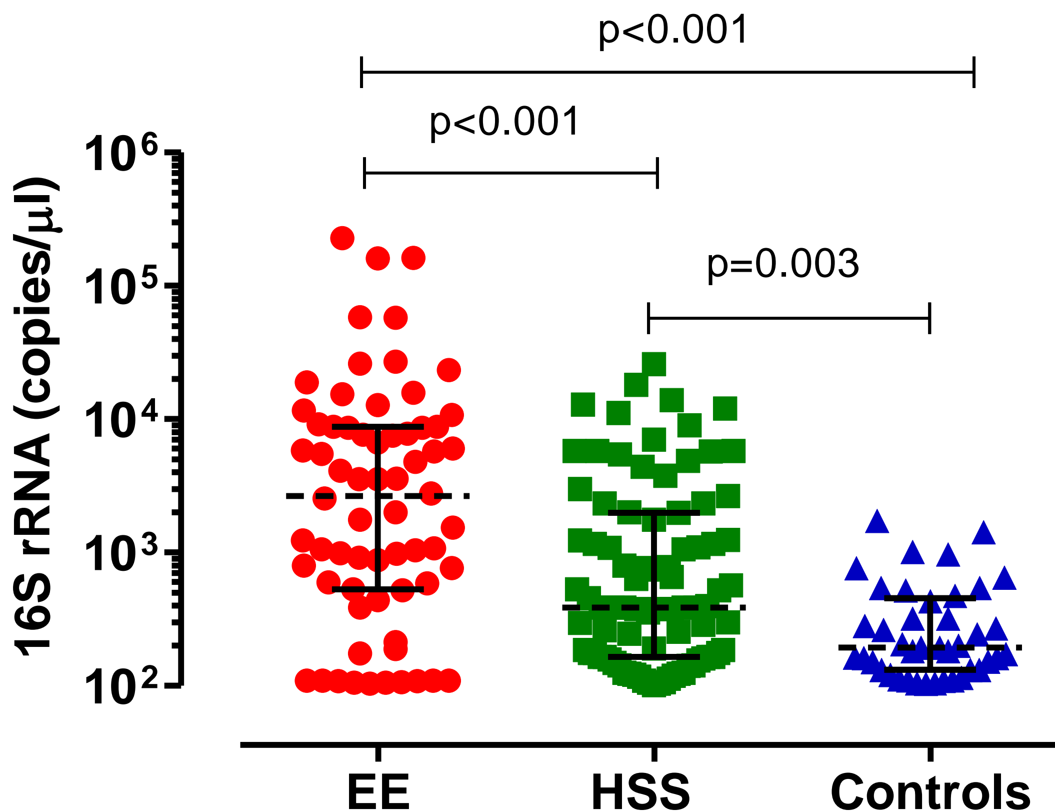


Figure 4.2: Comparison of plasma 16S rRNA copy number among EE, HSS participants and healthy controls. The horizontal broken lines within the dot plots are medians.

4.3.3 Comparison of TLRLs activity among the EE, HSS and Healthy Controls

TLRLs activity was significantly higher in the EE participants, median was 0.51 (IQR, 0 – 0.89) OD at 630nm compared to HSS participants 0.13 (IQR, 0 – 0.41); p=0.01 or healthy controls 0.02 (IQR, 0 – 0.12); p=0.004. TLRLs was also significantly higher in HSS participants and healthy controls (p=0.02) as shown in Figure 4.3. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used to compare all pairs. The results are shown with significance where applicable (p< 0.05).

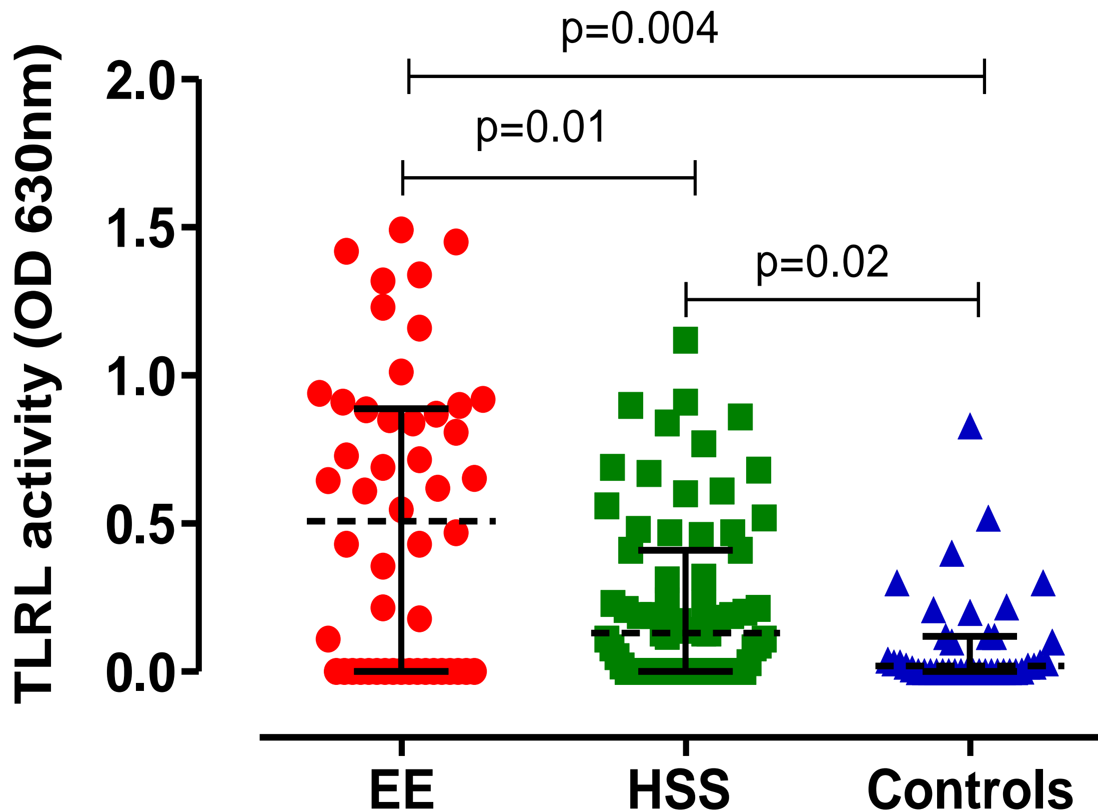


Figure 4.3: Comparison of TLRLs among EE, HSS participants and healthy controls measured from cell culture supernatant. The horizontal broken lines within the dot plots are medians.

4.4 Comparison of Host Immune Response to Microbial Translocation

Objective 2: To determine host immune responses to microbial translocation in individuals with environmental enteropathy, hepatosplenic shistosomiasis and healthy controls

4.4.1 Comparison of CRP levels among the EE, HSS and Healthy Controls

There was no statistical significant difference in CRP between participants with EE, median 6916 (IQR, 1197 – 15031) ng/ml compared to HSS participants 4373 (IQR, 1377 – 12932); p=0.09 or healthy controls 4156 (IQR, 763 – 7382); p=0.11. There was also no statistical significant

difference between HSS participants and healthy controls (p=0.24) as shown in Figure 4.4. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used to compare all pairs.

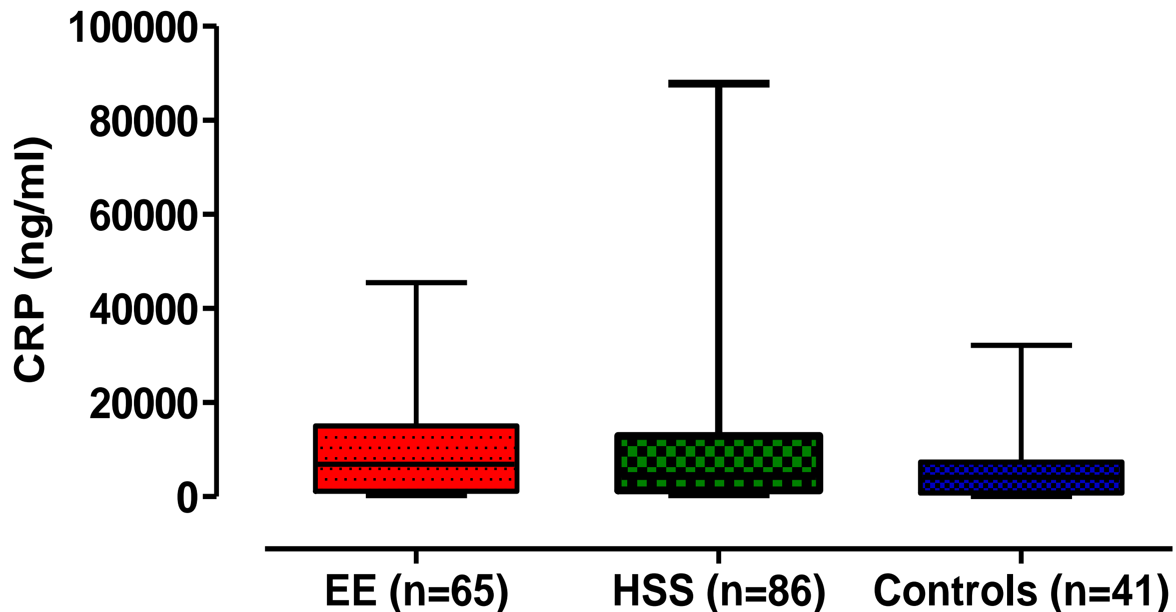


Figure 4.4 Comparison of plasma CRP among EE, HSS participants and healthy controls. The results were not statistically significant among the groups. The horizontal lines in the middle of the box and whiskers plots indicate medians.

4.4.2 Comparison of Plasma CD163 among the EE, HSS and Healthy Controls

Plasma CD163 was significantly higher levels in the EE participants, median 685.3 (IQR, 497.2 – 941.9) ng/ml compared to the healthy controls 421 (IQR, 274.3 – 663.5); p<0.001. It was also higher in the HSS participants 843 (IQR, 560.9 – 1176) compared to healthy controls (p<0.001). However, there was no significant difference between the EE and HSS participants (p=0.06) as shown in Figure 4.5. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used to compare all pairs. The results are shown with significance where applicable ($p < 0.05$).

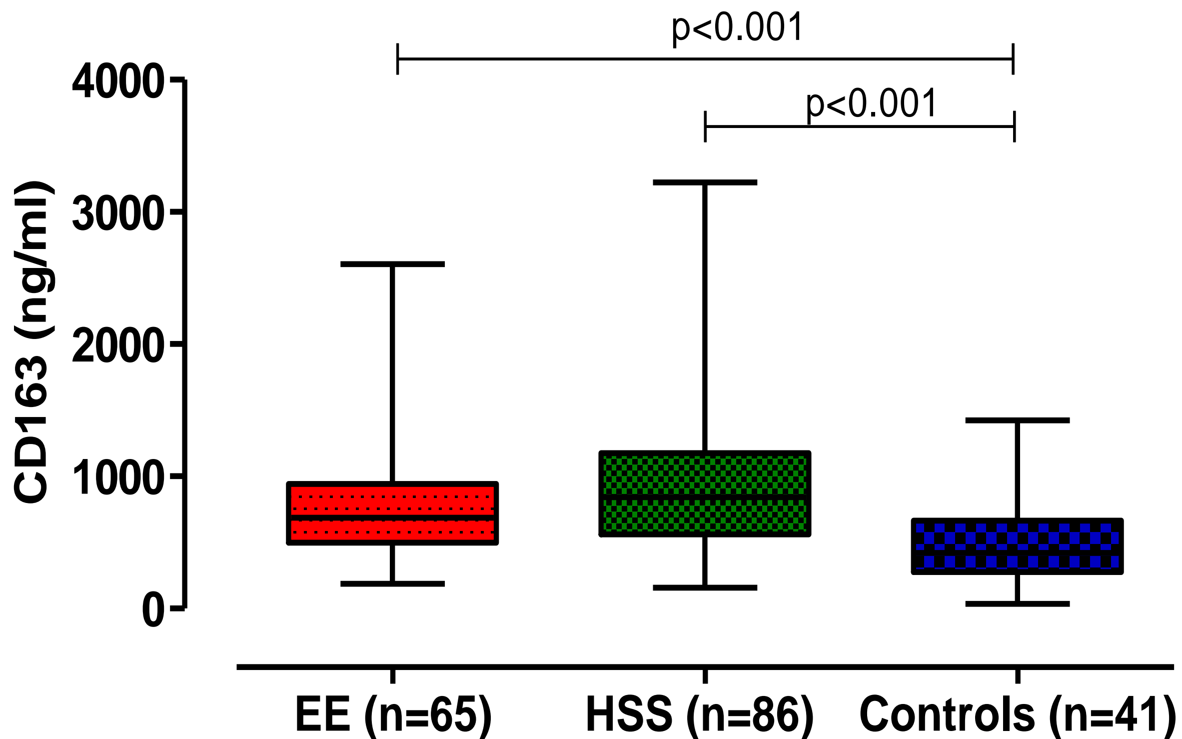


***Figure 4.5: Comparison of plasma CD163 concentration among EE, HSS participants and healthy controls. The horizontal lines in the middle of the box and whiskers plots indicate medians*.**

4.4.3 Comparison of Plasma Soluble CD14 levels among the EE, HSS and Healthy Controls

Soluble CD14 was significantly higher in participants with EE, median 1959 (IQR, 1582 – 2669) ng/ml compared to HSS participants 1712 (IQR, 1383 – 1964); p=0.01 or healthy controls 1170(IQR, 1045 – 1489); p<0.001. It was also significantly higher in the HSS participants compared to healthy controls (p<0.001) as shown in Figure 4.6. Kruskal-Wallis test was used to compare across the groups and Dunn’s post hoc test was used to compare all pairs. The results are shown with significance where applicable ($p < 0.05$).

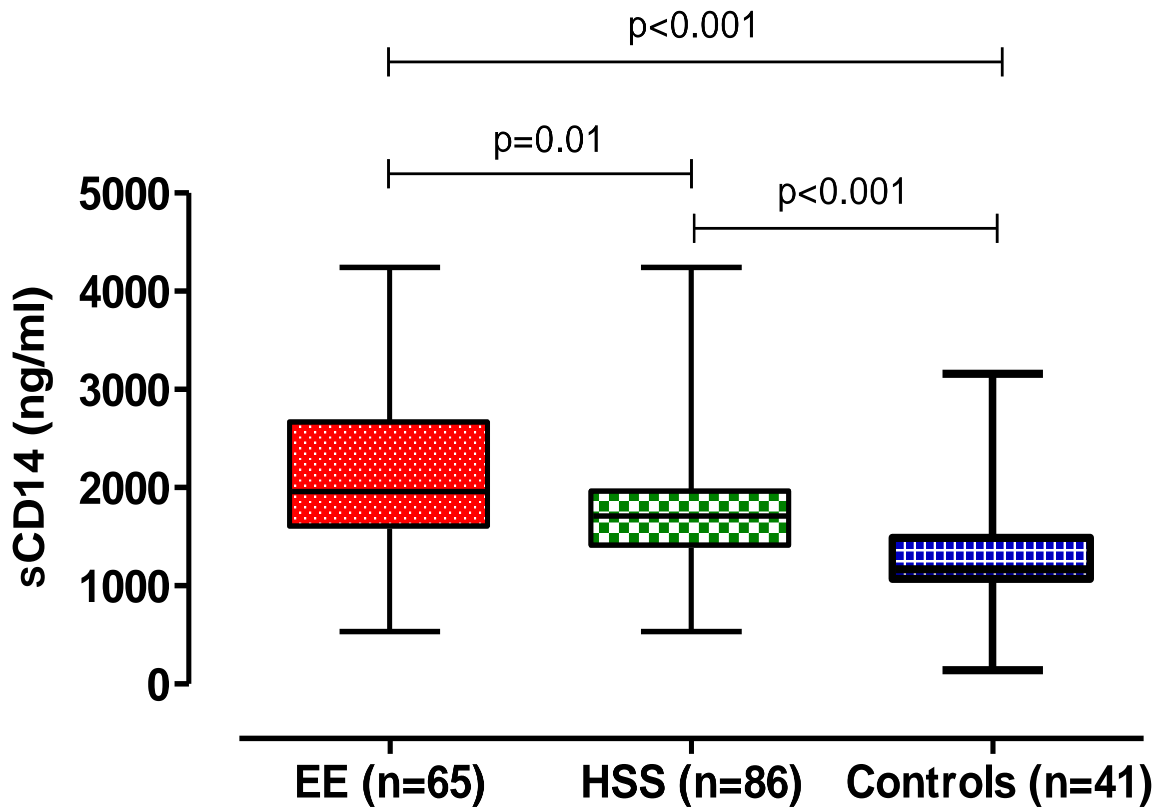


Figure 4.6: Comparison of plasma sCD14 among EE, HSS participants and healthy controls. The horizontal lines in the middle of the box and whiskers plots indicate medians.

4.4.4 Comparison of Plasma LBP among the EE, HSS and Healthy Controls

Plasma LBP was higher in both participants with EE, median 38.9 (IQR, 34.3 – 45.1) ng/ml and HSS 41.2 (IQR, 29.1 – 51.3) compared to the healthy controls 23.9 (IQR, 20.9 – 28.9); p<0.001. But there was no significant difference between the EE compared to the HSS participants (p=0.08) as shown in Figure 4.7. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used to compare all pairs. The results are shown with significance where applicable (p< 0.05).

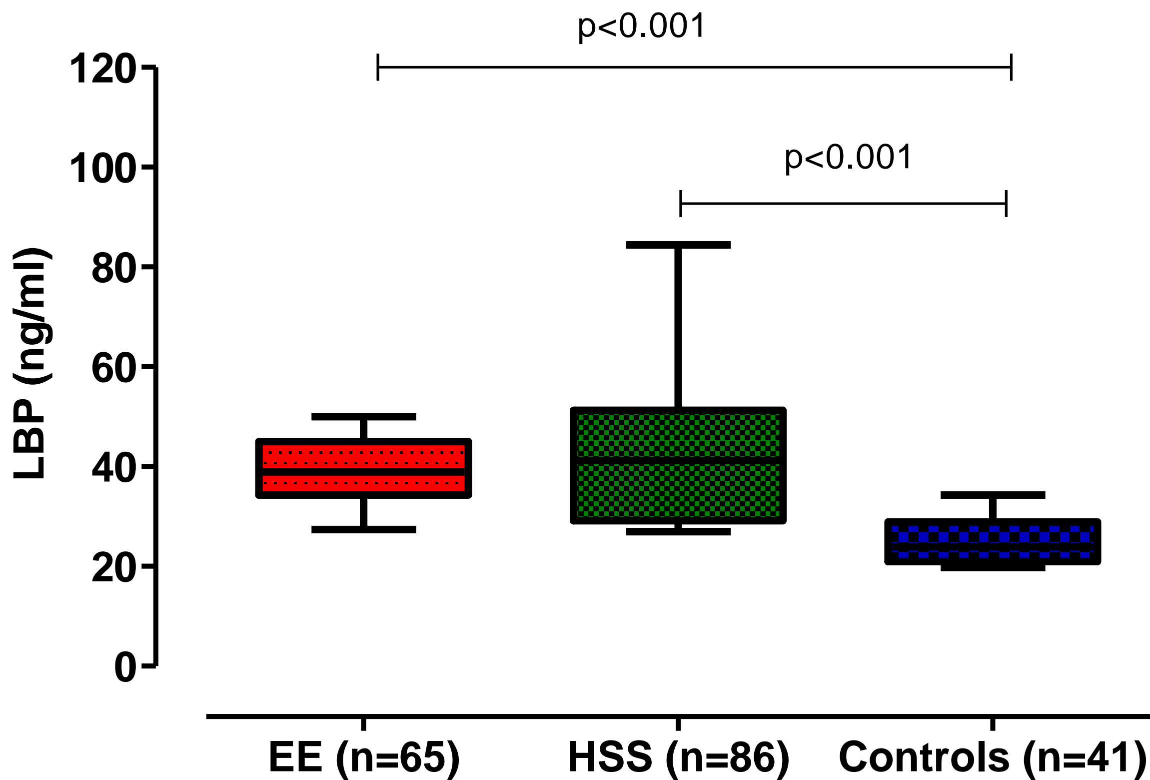


Figure 4.7: Comparison of plasma LBP among EE, HSS participants and healthy controls. The horizontal lines in the middle of the box and whiskers plots indicate medians.

Objective 3: To determine immune activation in individuals with environmental enteropathy, hepatosplenic schistosomiasis and healthy controls using cell culture *in-vitro* model.

4.5 Immune Activation in Response to Microbial Translocation

4.5.1 Comparison TNF-α among the EE, HSS and Healthy Controls as Measured from the Cell Culture Supernatant

TNF-α measured from cell culture supernatant was higher in participants with EE, median 444.5 (IQR, 48.3 – 869.5) pg/ml compared to HSS participants 88.9 (IQR, 27.4 – 460.5); p=0.003 or healthy controls 32.6 (IQR, 21.2 – 120.7); p<0.001. It was also significantly elevated in the HSS

participants compared to healthy controls (p=0.009) as shown in Figure 4.8. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used to compare all pairs. The results are shown with significance where applicable ($p < 0.05$).

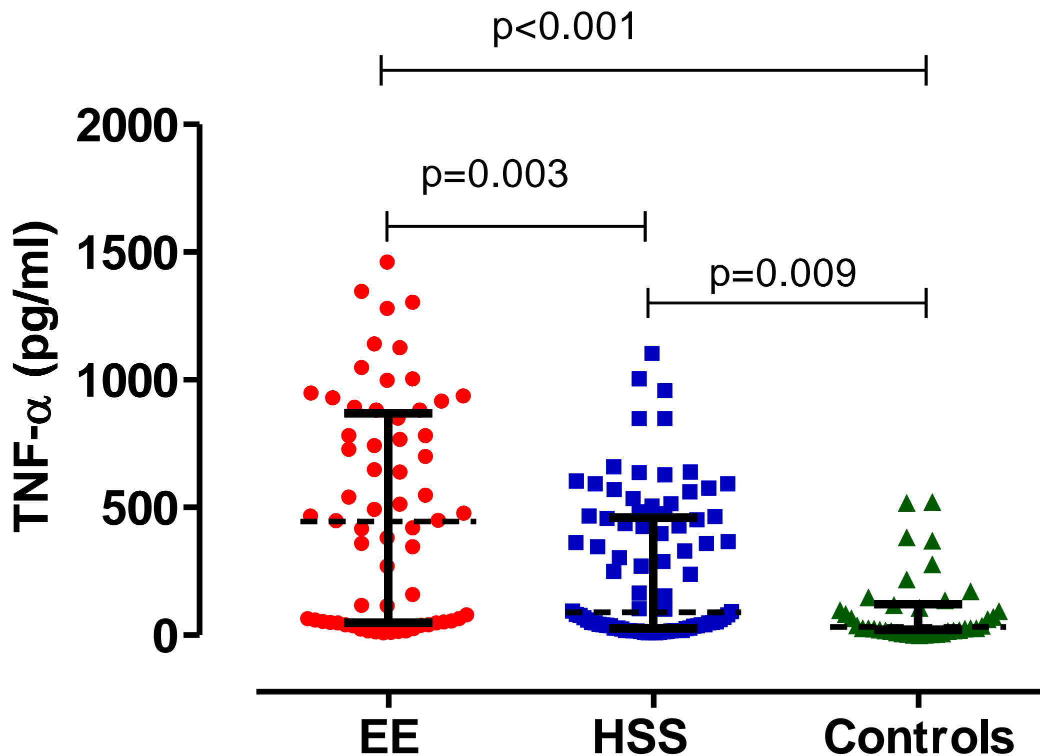


Figure 4.8: Comparison of TNF-α among EE, HSS participants and healthy controls measured from cell culture supernatant. The horizontal broken lines in the dot plots represent medians.

4.5.2 Comparison of IL-6 among the EE, HSS and Healthy Controls Measured from Cell Culture Supernatant

IL-6 was significantly higher in participants with EE, median 133.3 (IQR, 32.8 – 503.2) pg/ml compared to the HSS participants 65.3 (IQR, 21.3 – 358.3); p=0.01 or healthy controls 32.3 (IQR, 14.3 – 80.8); p<0.001. There was also significantly elevated IL-6 in the HSS participants compared to healthy controls (p=0.02) as shown in Figure 4.9. Kruskal-Wallis test was used to compare

across the groups and Dunn's post hoc test was used to compare all pairs. The results are shown with significance where applicable (p< 0.05).

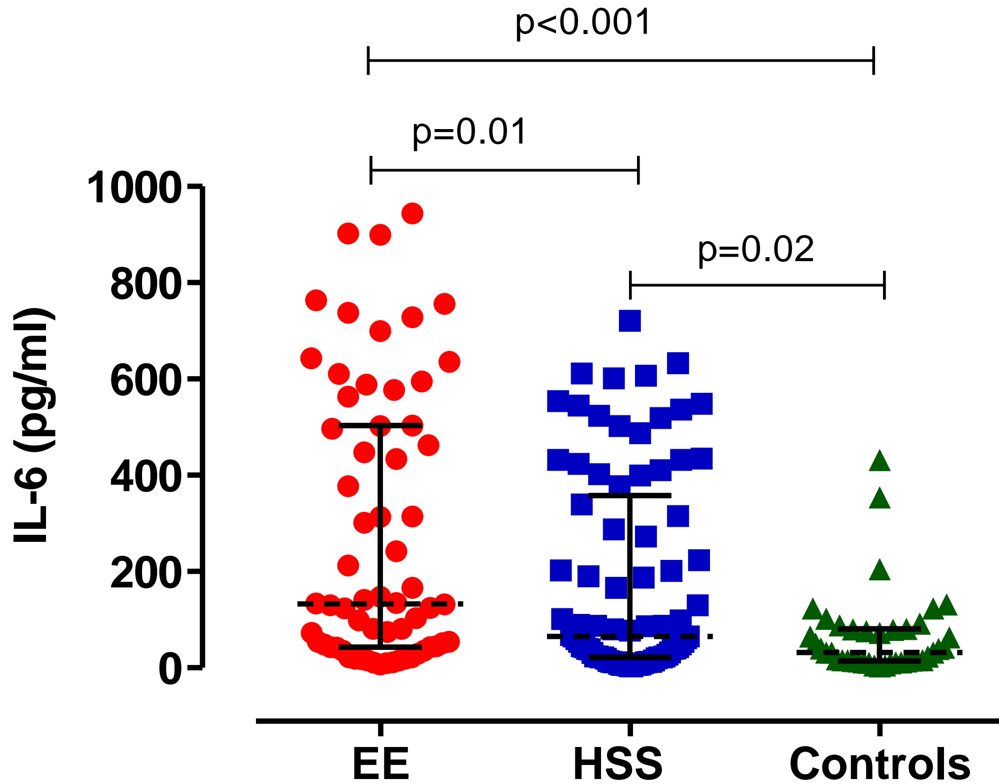


Figure 4.9: Comparison of IL-6 among EE, HSS participants and healthy controls measured from cell culture supernatant. The horizontal broken lines in the dot plots represent medians.

4.5.3 Comparison of IL-10 among EE, HSS and Healthy Controls Measured from Cell Culture Supernatant

IL-10 was significantly higher in participants with EE, median 112.2 (IQR, 10.6 – 388.2) pg/ml compared to HSS participants 37.5 (IQR, 11.3 – 233.5); p=0.009 or healthy controls 12.9 (IQR, 8.6 – 29.5); p=0.001. It was also significantly higher in HSS participants compared to healthy

controls (p=0.03) as shown in Figure 4.10. Kruskal-Wallis test was used to compare across the groups and Dunn's post hoc test was used to compare all pairs. The results are shown with significance where applicable ($p < 0.05$).

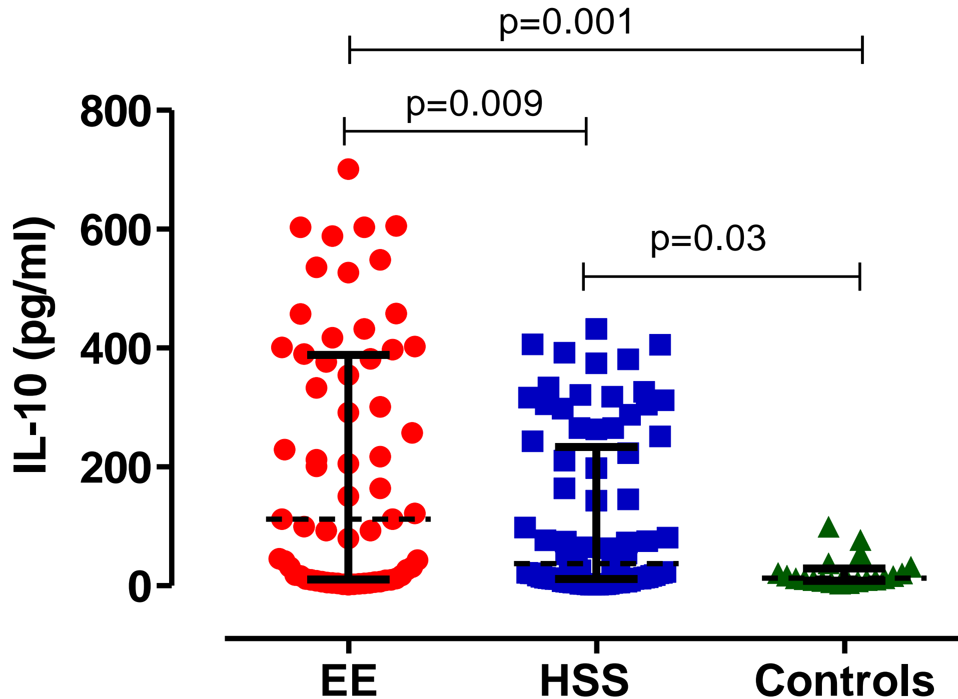


***Figure 4.10: Comparison of IL-10 among EE, HSS participants and healthy controls measured from cell culture supernatant. The horizontal broken lines in the dot plots represent medians*.**

4.5.4 Comparison of IL-4 among the EE, HSS and Healthy Controls Measured from Cell Culture Supernatant

IL-4 was significantly higher in participants with EE, median 7.4 (IQR, 5.4 – 10.9) pg/ml compared to HSS participants 4.9 (IQR, 3.4 – 7.4); p<0.001 or healthy controls 3.2 (IQR, 2.2 – 4.1); p<0.001. IL-4 was also significantly higher in HSS participants compared to healthy controls (p<0.001) as shown in Figure 4.11. Kruskal-Wallis test was used to compare across the groups and Dunn's post

hoc test was used to compare all pairs. The results are shown with significance where applicable ($p < 0.05$).

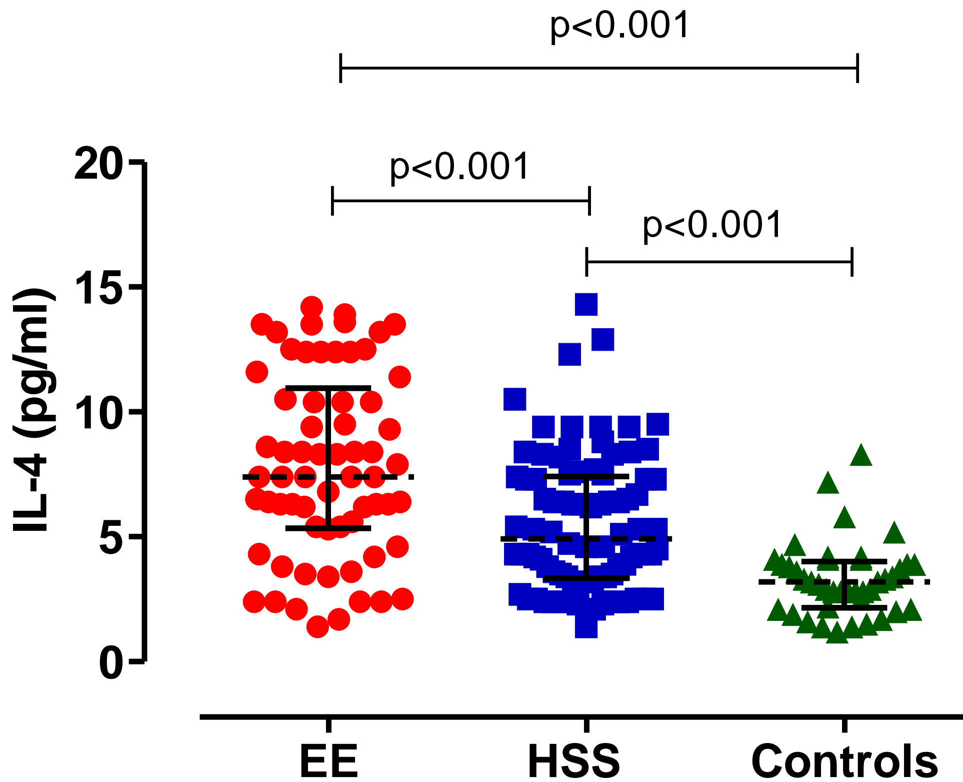


Figure 4.11: Comparison of IL-4 among EE, HSS participants and healthy controls measured from cell culture supernatant. The horizontal broken lines in the dot plots represent medians.

4.5.5 Comparison of IL-2 among the EE, HSS and Healthy Control Measured from Cell Culture Supernatant

IL-2 was significantly elevated in participants with EE, median 3.9 (IQR, 2.8 – 5) compared to HSS participants 3.2 (IQR, 2.1 – 4.9); p=0.02 or healthy controls 2.2 (IQR, 1.7 – 2.8); p<0.001. IL-2 was also significantly higher in HSS participants compared to healthy controls (p=0.007) as shown in Figure 4.12. Kruskal-Wallis test was used to compare across the groups and Dunn's post

hoc test was used to compare all pairs. The results are shown with significance where applicable ($p < 0.05$).

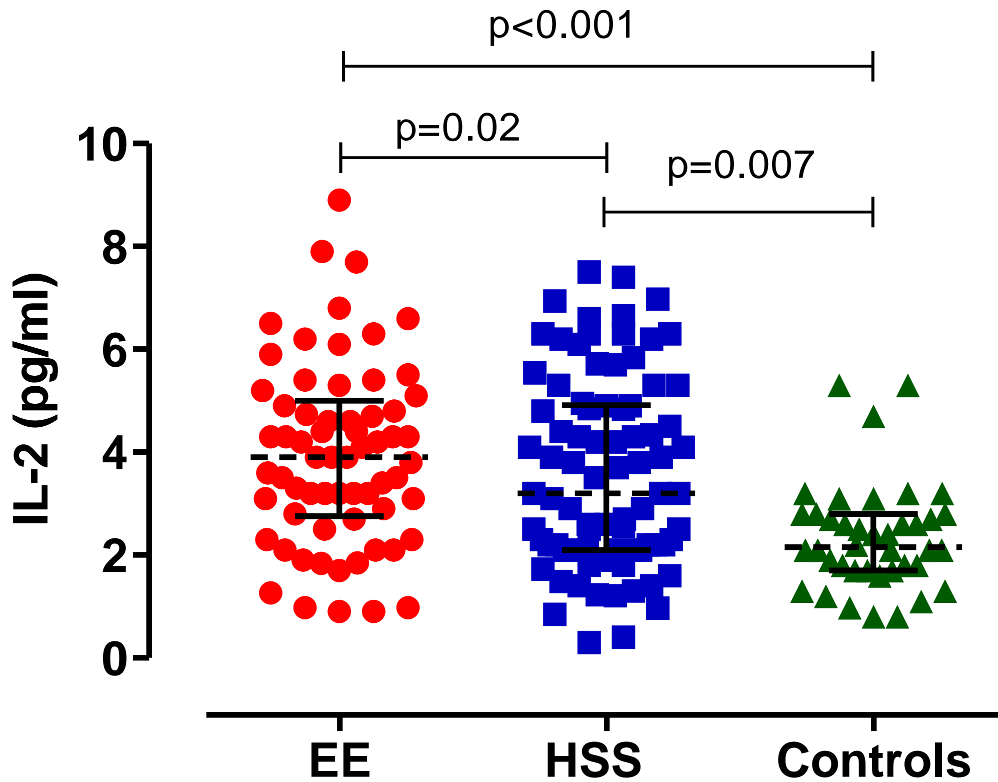


Figure 4.12: Comparison of IL-2 among EE, HSS participants and healthy controls measured from cell culture supernatant. The horizontal broken lines in the dot plots represent medians.

Specific Objective IV: To determine the relationship among plasma direct biomarkers of microbial translocation and immune activation in environmental enteropathy and hepatosplenic schistosomiasis in cell culture supernatant.

4.6 Relationship between Direct Biomarkers and Host Immune Response to Microbial Translocation

4.6.1 Relationship between Direct Biomarkers and Host Immune Response to Microbial Translocation in Participants with EE

To determine the relationship between direct biomarkers of microbial translocation and host response to microbial translocation, correlation matrices were conducted for each group of participants. In the EE participants, there was positive and significant correlations between LPS with sCD14 ($r=0.37$; $p<0.05$) and with LBP ($r=0.39$; $p<0.05$). There was also a positive and significant correlation between 16S rRNA with LBP ($r=0.29$; $p<0.05$). There was positive and significant correlations between TLRLs with sCD14 ($r=0.29$; $p<0.05$), sCD163 ($r=0.28$; $p<0.05$) and LBP ($r=0.41$; $p<0.01$). There was also positive and significant correlations among direct biomarkers LPS with 16S rRNA ($r=0.52$; $p<0.01$), TLRLs ($r=0.43$; $p<0.01$) and 16S rRNA with TLRLs ($r= 0.61$; $p<0.01$). There was positive and significant relationship among biomarkers of host response to microbial translocation for sCD14 with LBP ($r=0.31$; $p<0.01$) and sCD163 ($r=0.27$; $p<0.05$) as shown in Table 4.2.

4.6.3: Relationship between Direct Biomarkers and Host Response to Microbial Translocation in the Healthy Controls

In the healthy control group, there was only positive and significant correlation between TLRLs and LBP ($r=0.28$; $p<0.05$) as shown in Table 4.4.

Table 4.2: Correlation matrices of direct biomarkers of microbial translocation and biomarkers of host response to microbial translocation in individuals with environmental enteropathy

Environmental enteropathy (n=67)							
Variable	LPS	16S rRNA	TLRL	CRP	sCD14	sCD163	LBP
LPS	1.0000						
16S rRNA	0.5292**	1.0000					
TLRL	0.4376**	0.6171**	1.0000				
CRP	0.0167	0.0477	0.0667	1.0000			
sCD14	0.3734*	0.0577	0.2907*	0.0708	1.0000		
sCD163	0.1154	0.1398	0.2876*	0.0722	0.2704*	1.0000	
LBP	0.3972**	0.2982*	0.4101**	0.1102	0.3132**	0.2114	1.0000

Two tailed correlations * p < 0 .05, ** p < 0 .01

4.6.2: Relationship between Direct Biomarkers and Host Response to Microbial Translocation in participants with HSS

There was positive and significant correlations between LPS with LBP (r=0.34; p<0.01), 16S rRNA with LBP (r=0.23; p<0.05), TLRLs with LBP (r=0.43; p<0.01) and sCD14 (r=0.26; p<0.05). There was positive and significant correlations among direct biomarkers of microbial translocation, LPS with 16S rRNA (r=0.51; p<0.01), TLRLs (r=0.33; p<0.01) and 16S rRNA with TLRLs (r=0.62; p<0.01). There was also positive and significant relationship between sCD14 and LBP (r=0.24; p<0.05). Where there are positive and significant correlations between direct biomarkers of microbial translocation and biomarkers of host response to microbial translocation as shown in Table 4.3.

Table 4.3: Correlation matrices showing relationships between direct biomarkers of microbial translocation and host immune response to microbial translocation in HSS participants

Hepatosplenic schistosomiasis participants (n=86)							
Variable	LPS	16S rRNA	TLRLs	CRP	sCD14	sCD163	LBP
LPS	1.0000						
16S rRNA	0.5163**	1.0000					
TLRL	0.3392*	0.6254**	1.0000				
CRP	0.1550	0.1391	0.1737	1.0000			
sCD14	0.1070	0.0157	0.2622*	0.0632	1.0000		
sCD163	0.1134	0.0996	0.2131	0.0076	0.1285	1.0000	
LBP	0.3442**	0.2328*	0.4321**	0.1012	0.2456*	0.1998	1.0000

Two tailed correlations * p < 0 .05, ** p < 0 .01

Table 4.4: Correlation matrix showing relationship between biomarkers of microbial origin and biomarkers of host response to microbial translocation in healthy controls

Healthy controls (n=41)							
Variable	LPS	16S rRNA	TLRLs	CRP	sCD14	sCD163	LBP
LPS	1.0000						
16S rRNA	0.0219	1.0000					

TLRL	0.2093	0.0658	1.0000				
CRP	0.1104	0.2218	0.0756	1.0000			
sCD14	0.1896	0.0615	0.0440	0.0476	1.0000		
sCD163	0.0752	0.0018	0.1217	0.1261	0.1329	1.0000	
LBP	0.2101	0.1753	0.2871*	0.1010	0.1765	0.0999	1.0000

Two tailed correlations * p < 0 .05, ** p < 0 .01

4.6.4: Relationship between Direct Biomarkers and Host Response to Microbial Translocation in participants with EE and HSS Combined

There was positive and significant correlations of LPS with sCD14 (r=0.39; p<0.01), sCD163 (r=0.27; p<0.05), and LBP (r=0.42; p<0.01). The correlations were also positive and significant for 16S rRNA with CRP (r=0.27; p<0.05) and LBP (r=0.39; p<0.01). For TLRLs the correlations were positively and significantly with sCD14 (r=0.31; p<0.05), CRP (r=0.23; p<0.05), sCD163 (r=0.31; p<0.05) and LBP (r=0.44; p<0.01). There was also positively and significant correlations among biomarkers of host immune response, sCD14 with sCD163 (r=0.35; p<0.01) and LBP (r=0.31; p<0.31). In summary, after combining the EE and HSS participants, there was greater and significant correlations compared to any other group as shown in Table 4.5.

Table 4.5: Correlation matrices showing relationships between biomarkers of microbial origin and biomarkers of host response to microbial translocation in environmental enteropathy and hepatosplenic schistosomiasis participants combined

EE group and HSS participants combined (n=153)

Variable	LPS	16S rRNA	TLRL	CRP	sCD14	sCD163	LBP
LPS	1.0000						
16S rRNA	0.5231**	1.0000					

TLRL	0.6104**	0.6404**	1.0000				
CRP	0.0407	0.2704*	0.2308*	1.0000			
sCD14	0.3922**	0.1693	0.3112*	0.10125*	1.0000		
sCD163	0.2751*	0.1077	0.3126*	0.0541	0.3582**	1.0000	
LBP	0.4213**	0.3999**	0.4409**	0.1301	0.3176*	0.2211	1.0000

Two tailed correlations * p < 0 .05, ** p < 0 .01

4.7 Relationship between Cytokines Measured from Cell Culture Supernatant and Plasma Direct Biomarkers of Microbial Translocation

4.7.1 Correlations between TNF-α and LPS in participants with EE

In participants with EE, TNF-α was positively and significantly correlated to LPS ($r = 0.61$; $p<0.001$) as shown (Figure 4.13).

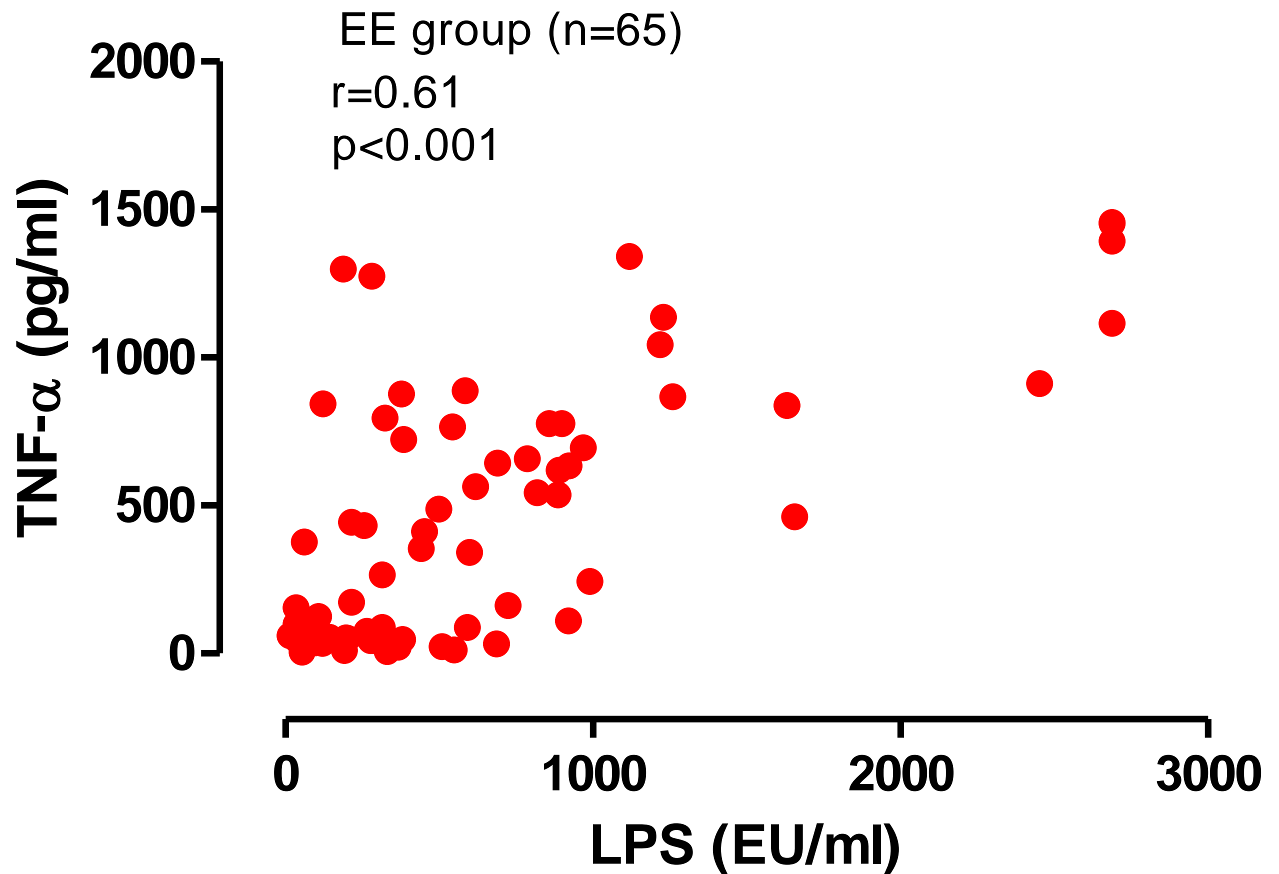


Figure 4.13: Correlation between TNF- α and lipopolysaccharides in the EE group. There was positive and significant correlation between TNF- α and lipopolysaccharides. Spearman's correlation is shown.

4.7.2: Correlation between TNF-α and LPS in participants with HSS

In participants with HSS, there was positive and significant correlation between TNF-α and LPS ($r = 0.45$; $p<0.001$) as shown (Figure 4.14).

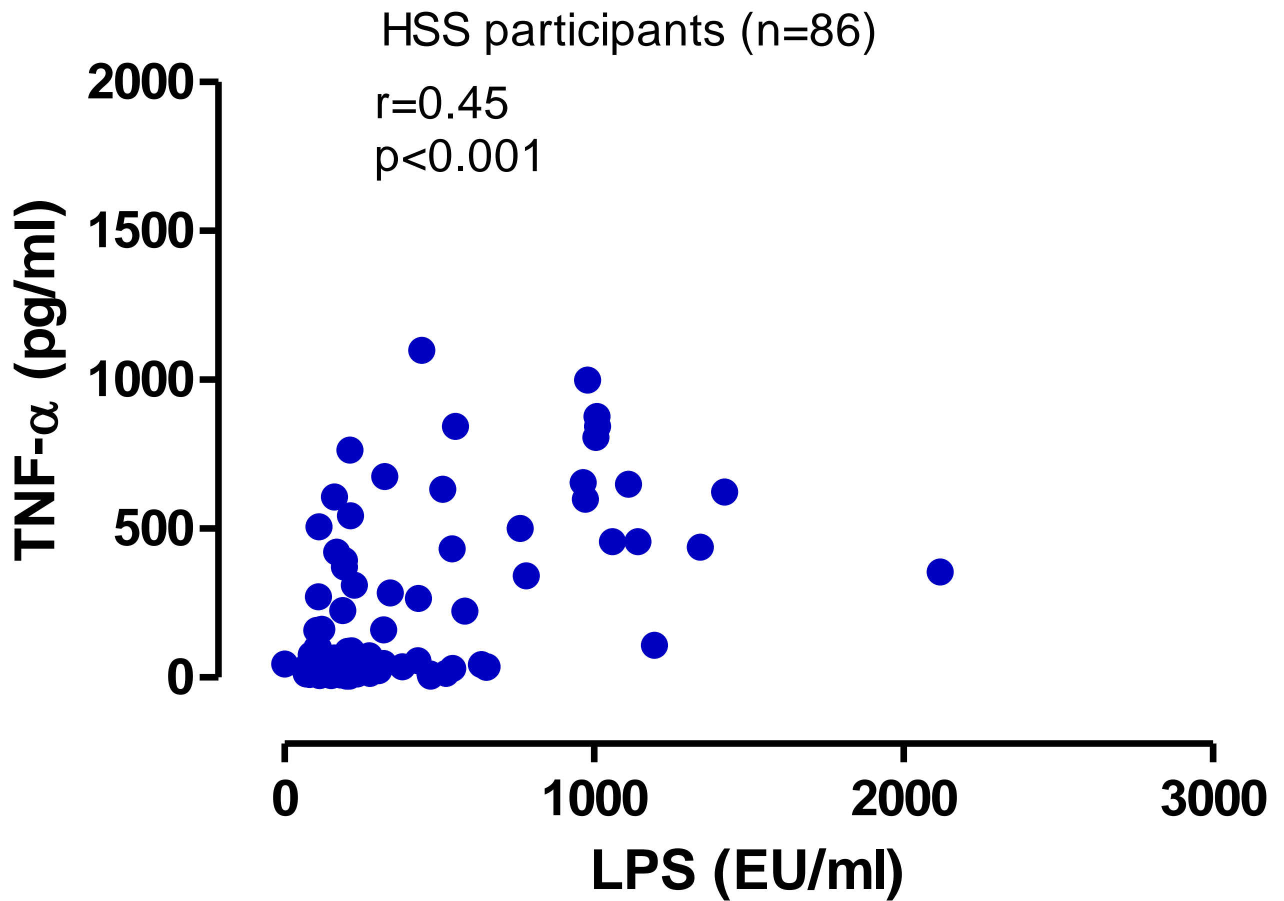


Figure 4.14: Correlation between TNF- α and lipopolysaccharides in the EE group. There was positive and significant correlation between TNF-α and lipopolysaccharides. Spearman's correlation is shown.

4.7.3: Correlation between TNF-α and LPS in Healthy Controls

In healthy controls, there was positive and but non-significant correlation between TNF-α and LPS (r= 0.30; p=0.05) as shown (Figure 4.15).

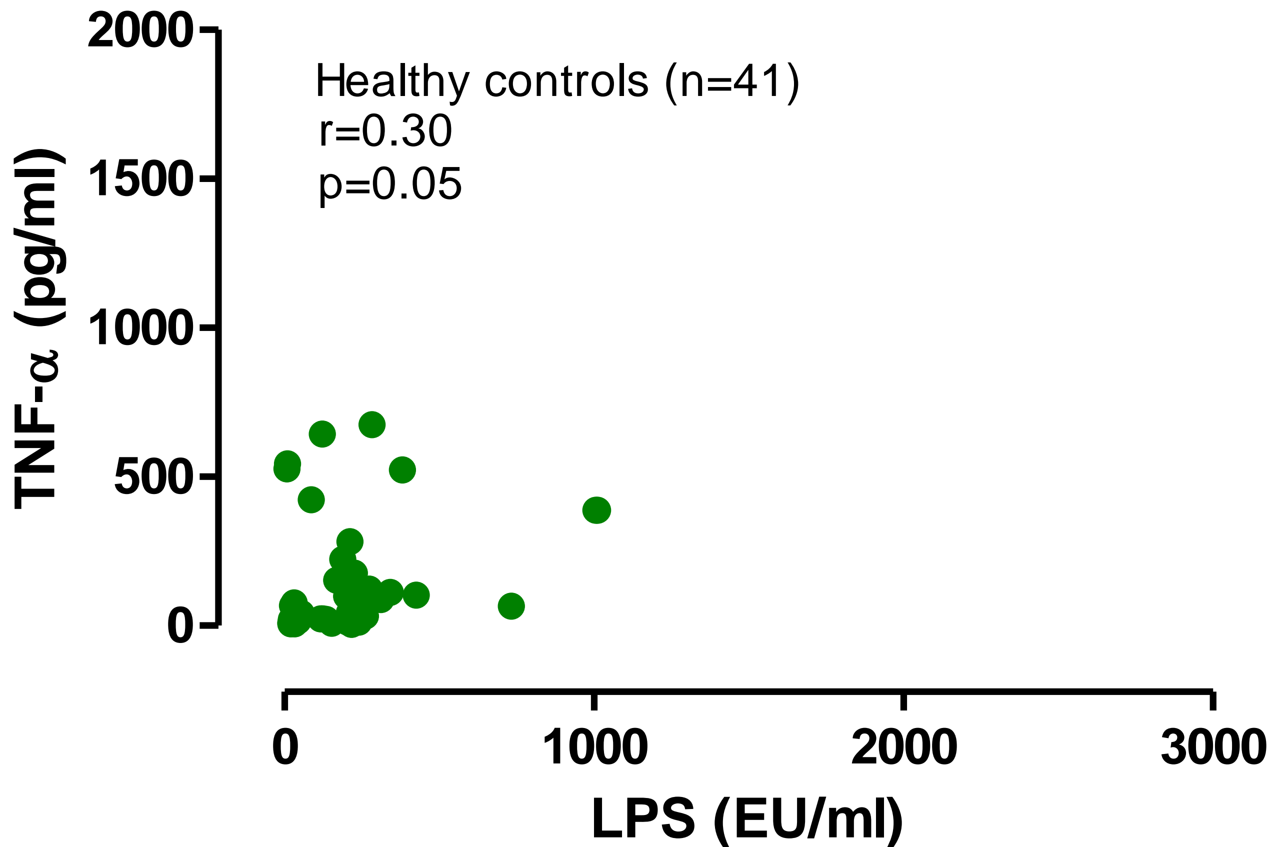


Figure 4.15: Correlation between TNF- α and lipopolysaccharides in healthy controls. There positive but non-significant correlation between TNF- α and lipopolysaccharides. Spearman's correlation is shown.

4.7.4: Correlation between TNF-α and 16S rRNA copy number in participants with EE

In participants with EE, there was positive and significant correlation between TNF-α and 16S rRNA copy number (r= 0. 53; $p<0.0001$) as shown (Figure 4.16).

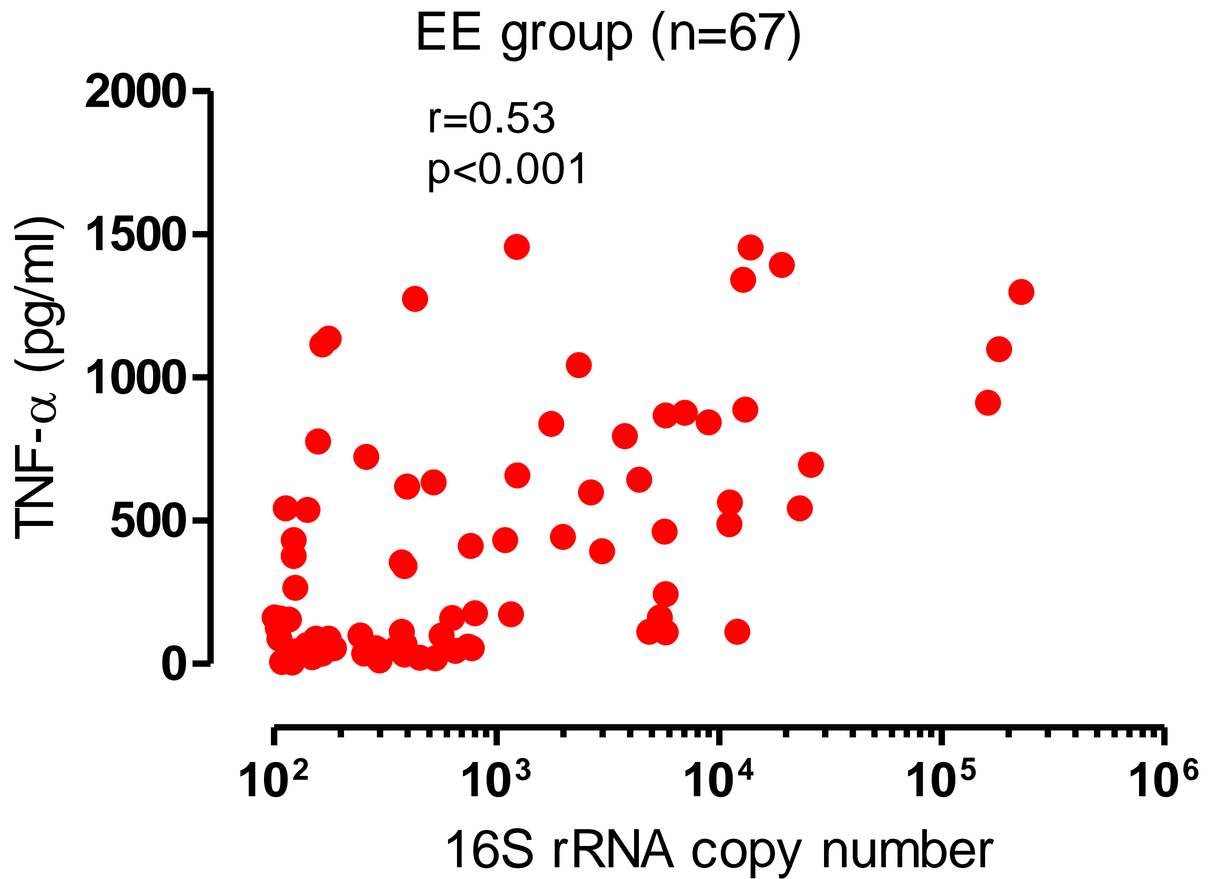


Figure 4.16: Correlation between TNF-α and 16S rRNA copy number in participants with EE. There was positive and significant correlation between TNF-α and 16S rRNA copy number. Spearman's correlation is shown.

4.7.5: Correlation between TNF-α and 16S rRNA copy number in participants with HSS

In participants with HSS, there was positive and significant correlation between TNF-α and 16S rRNA copy number (r= 0. 42; p<0.001) as shown (Figure 4.17).

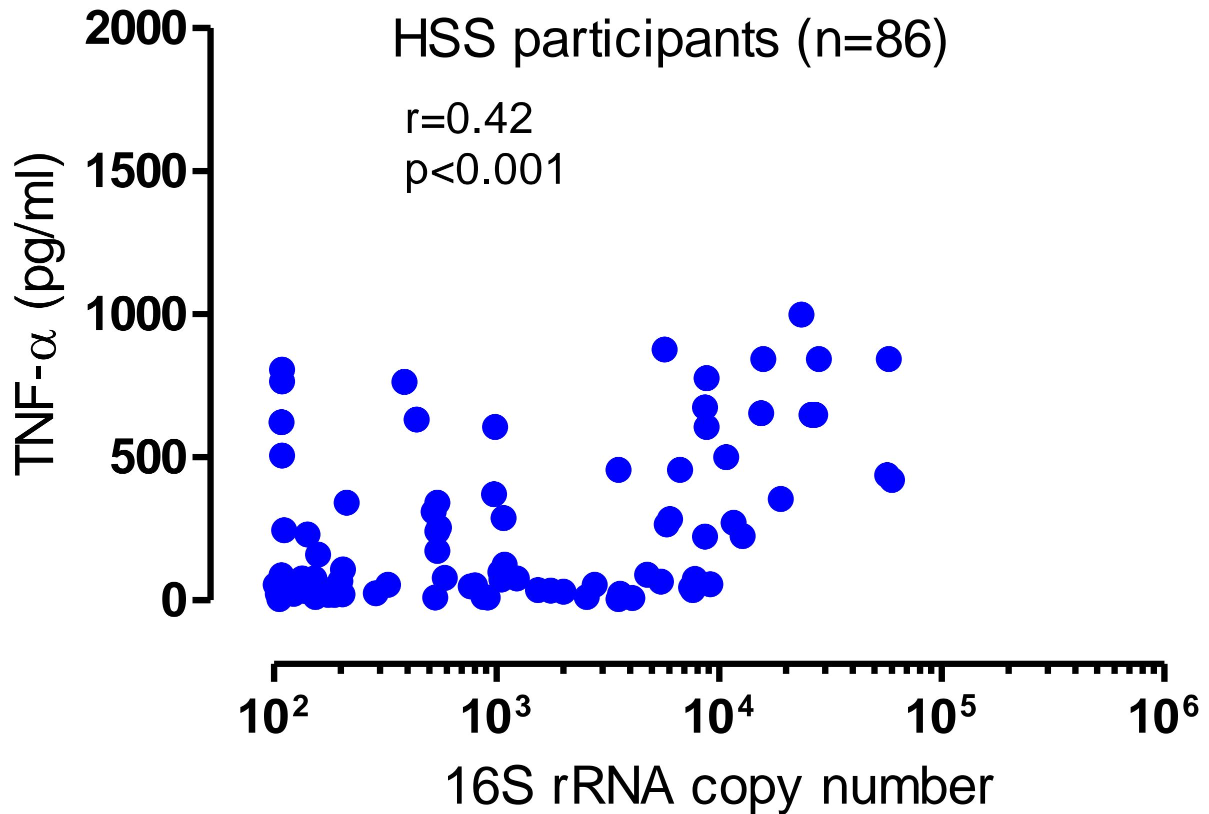


Figure 4.17: Correlation between TNF-α and 16S rRNA copy number in hepatosplenic schistosomiasis. There was positive and significant correlation between TNF-α and 16S rRNA copy number. Spearman's correlation is shown.

4.7.6 Correlation between TNF-α and 16S rRNA copy number in Health Controls

In healthy controls, there was positive but non-significant correlation between TNF-α and 16S rRNA copy number (r= 0. 29; p=0.06) as shown (Figure 4.18).

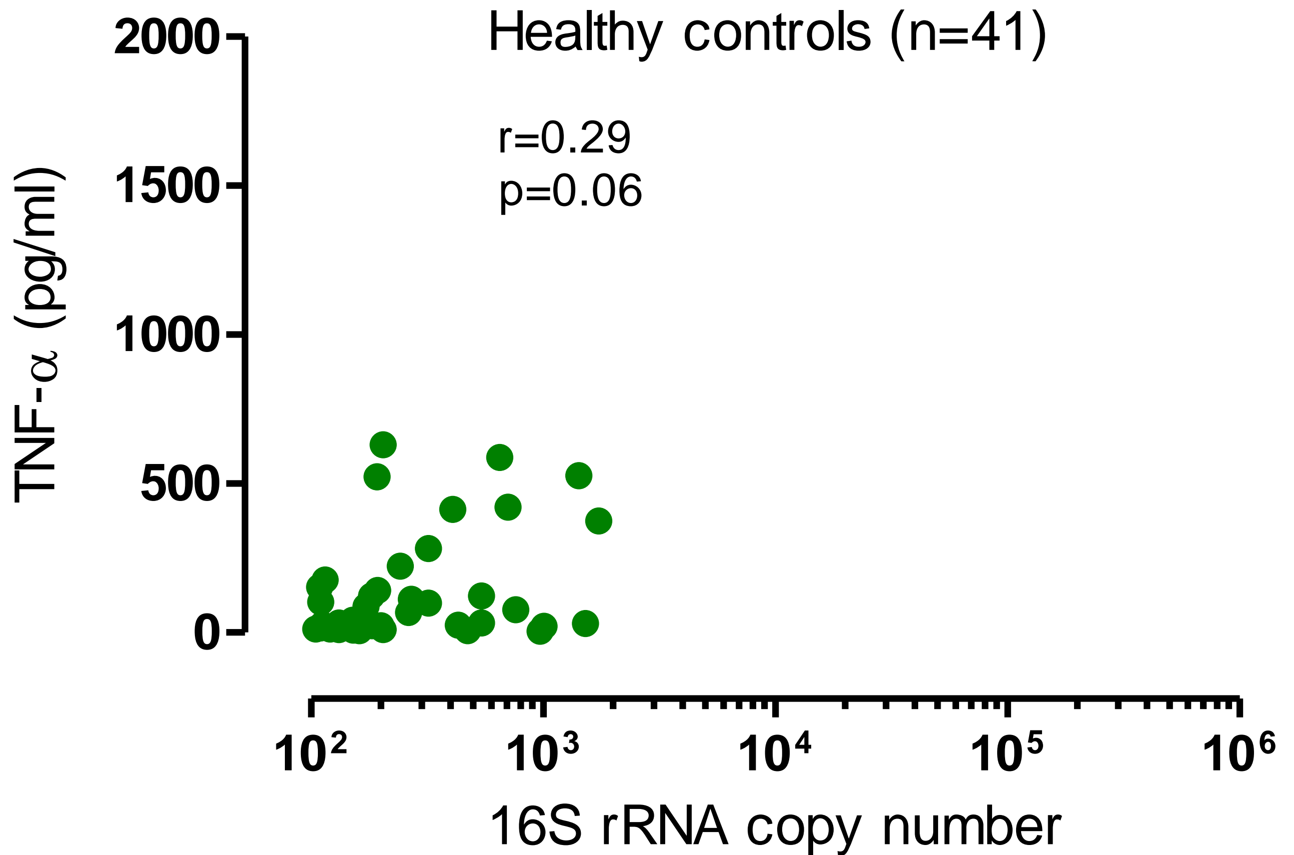


Figure 4.18: Correlation between TNF- α and 16S rRNA copy number in healthy controls. There was positive but non-significant correlation between TNF- α and 16S rRNA copy number. Spearman's correlation is shown.

4.7.7 Correlation between TNF-α and TLRLs activity in participants with EE

In participants with EE, there was positive and significant correlation between TNF-α and TLRLs activity (r= 0. 73; p<0.001) as shown (Figure 4.19).

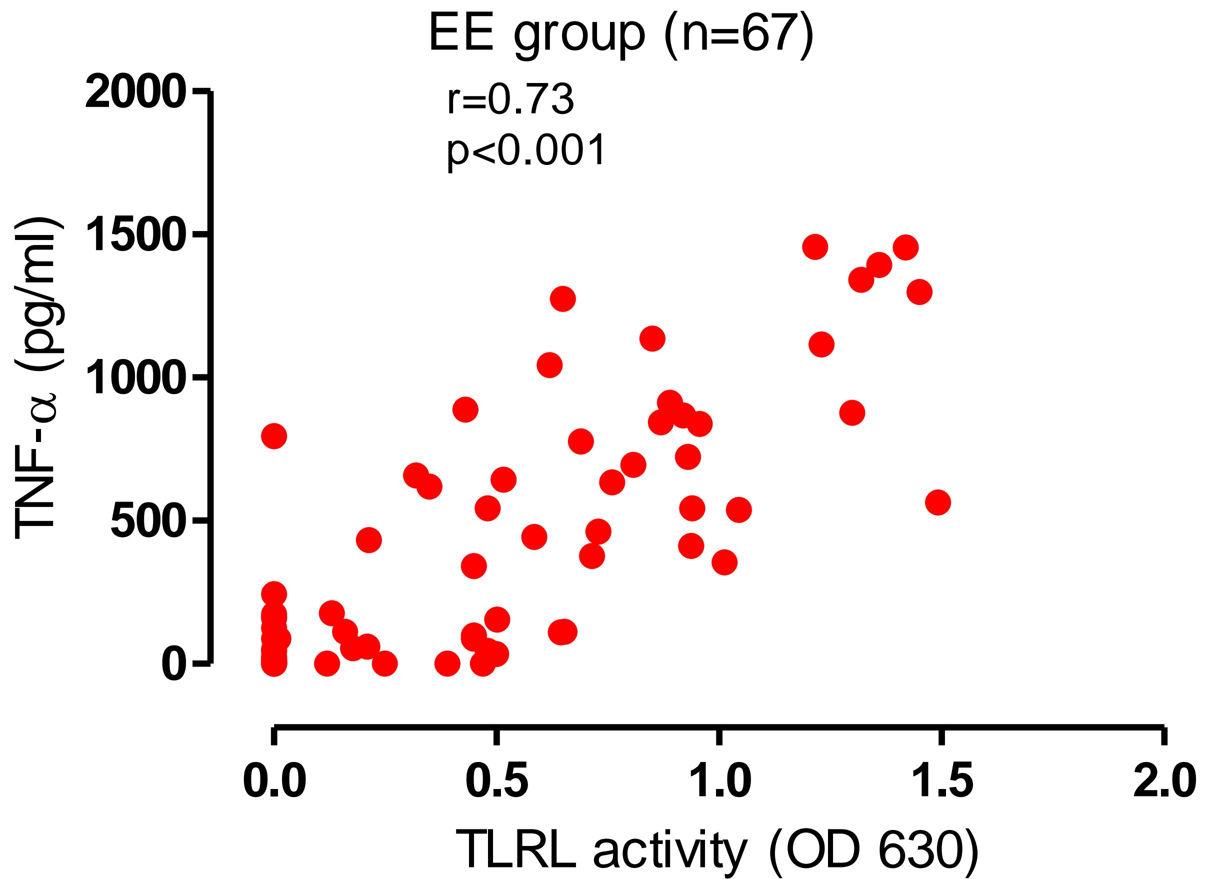


Figure 4.19: Correlation between TNF-α and Toll-like receptor ligands activity in participants with environmental enteropathy. There was positive and significant between TNF-α and Toll-like receptors. Spearman's correlation is shown.

4.7.8: Correlation between TNF-α and TLRLs activity in participants with HSS

In participants with HSS, there was positive and significant correlation between TNF-α and TLRLs activity (r= 0. 55; p<0.001) as shown (Figure 4.20).

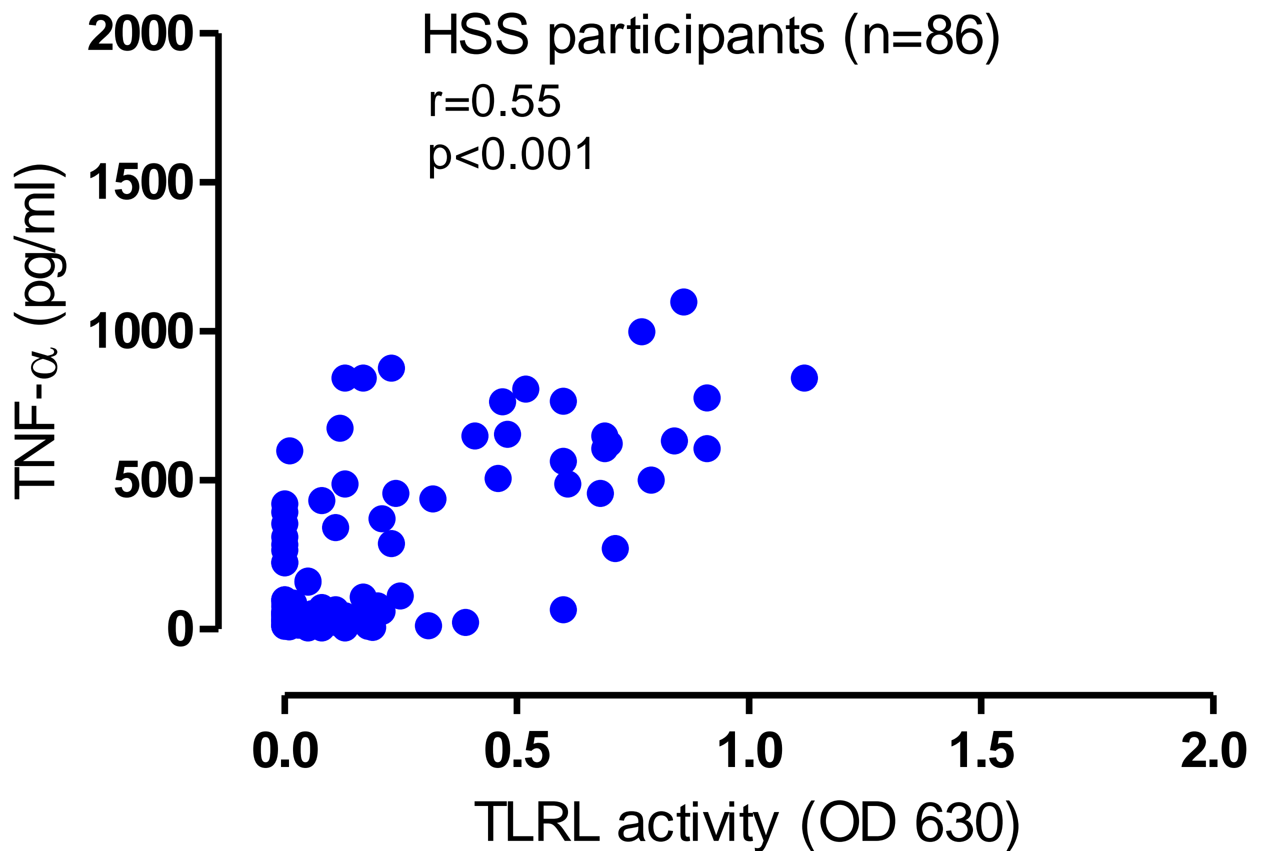


Figure 4.20: Correlation between TNF-α and Toll-like receptor ligand activity in participants with hepatosplenic schistosomiasis. There was positive and significant correlation between TNF-α and Toll-like receptors activity. Spearman's correlation is shown.

4.7.9 Correlation between TNF-α and TLRLs activity in Healthy Controls

In health controls, there was positive and significant correlation between TNF-α and TLRLs activity (r= 0. 33; p=0.04) as shown (Figure 4.20).

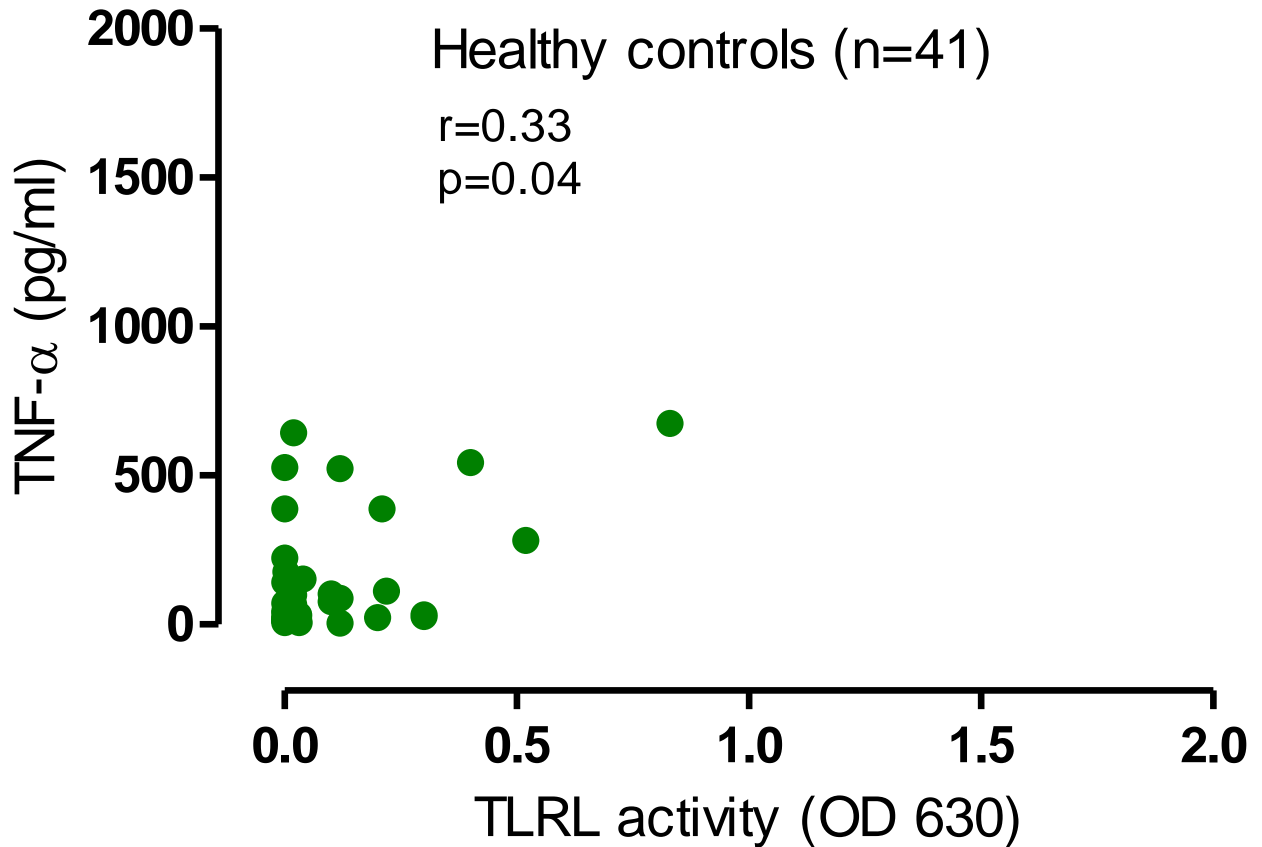


Figure 4.21: Shows correlation between TNF-α and Toll-like receptor ligands activity in healthy controls. There was positive and significant correlation between TNF- α and Toll-like receptor ligands activity. Spearman's correlations are shown.

4.8: Correlation between IL-6 and Direct Biomarkers of Microbial Translocation

4.8.1: Correlation between IL-6 and LPS in participants with EE

In participants with EE, there was positive and significant correlation between IL-6 and LPS (r= 0. 48; p<0.001) as shown (Figure 4.22).

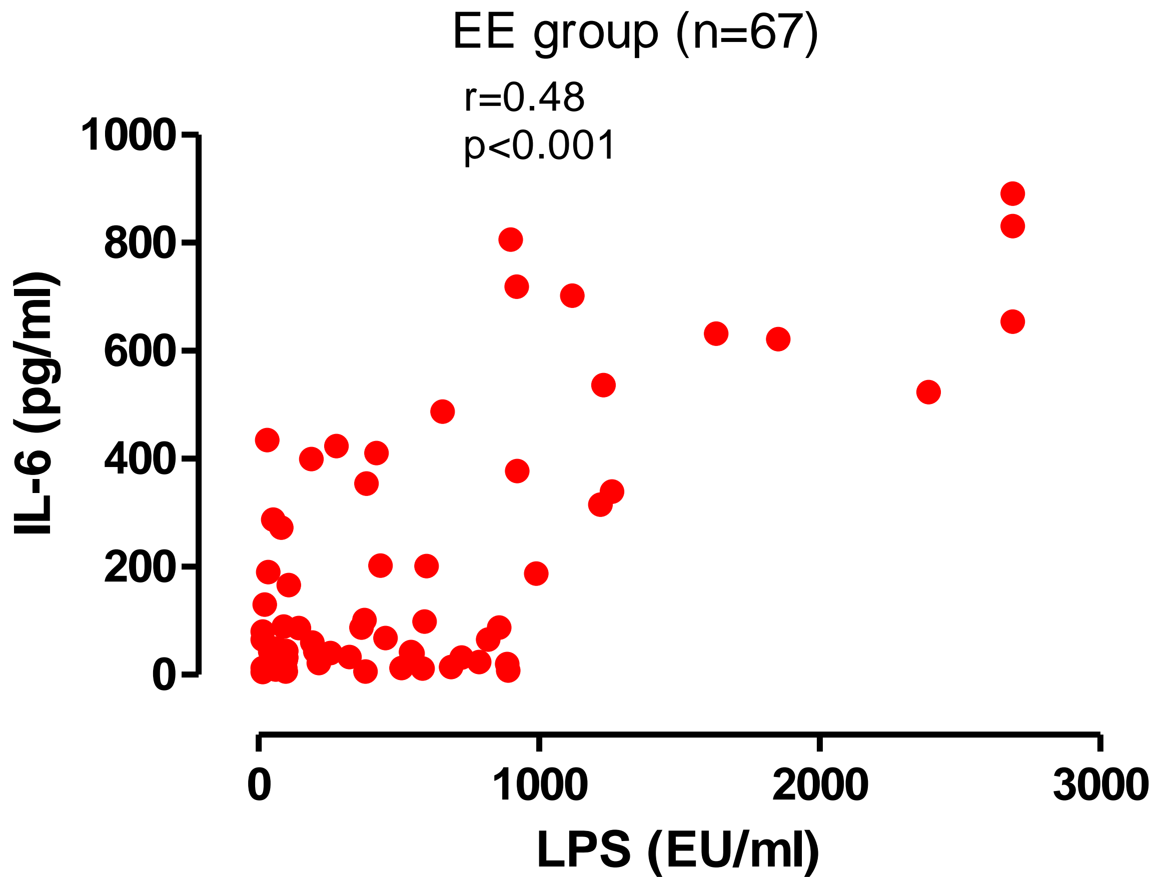


Figure 4.22: Correlation between IL-6 and lipopolysaccharides in participants with environmental enteropathy. There was positive and significant correlation between IL-6 and lipopolysaccharides. Spearman's correlation is shown.

4.8.2: Correlation between IL-6 and LPS in participants with HSS

In participants with HSS, there was positive and significant correlation between IL-6 and LPS (r= 0. 44; p<0.001) as shown (Figure 4.23).

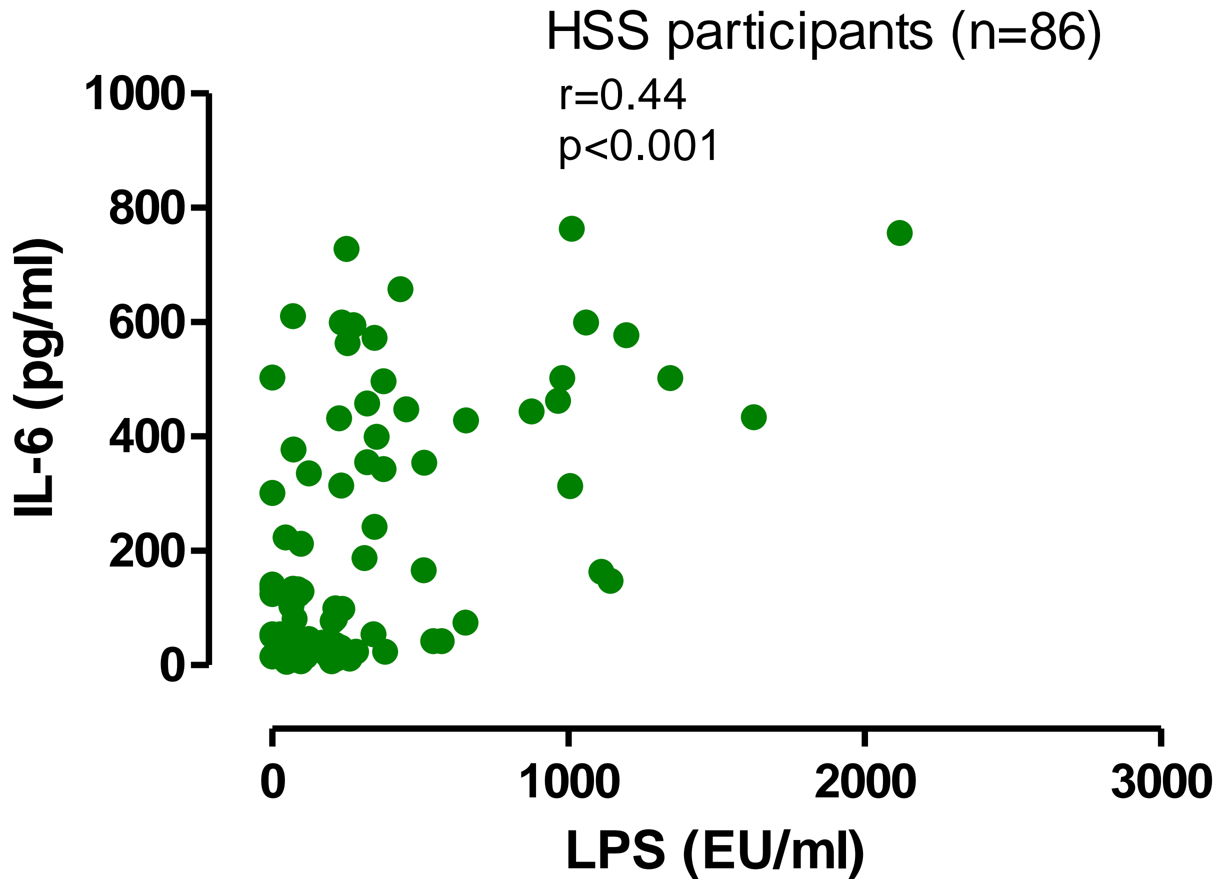


Figure 4:23: Shows correlation between IL-6 and LPS in participants with hepatosplenic shistosomiasis. There was positive and significant correlation between IL-6 and lipopolysaccharides. Spearman's correlation is shown.

4.8.3: Correlation between IL-6 and LPS in Healthy Controls

In healthy controls, there was positive but non-significant correlation between IL-6 and LPS (r= 0. 23; p=0.13) as shown (Figure 4.24).

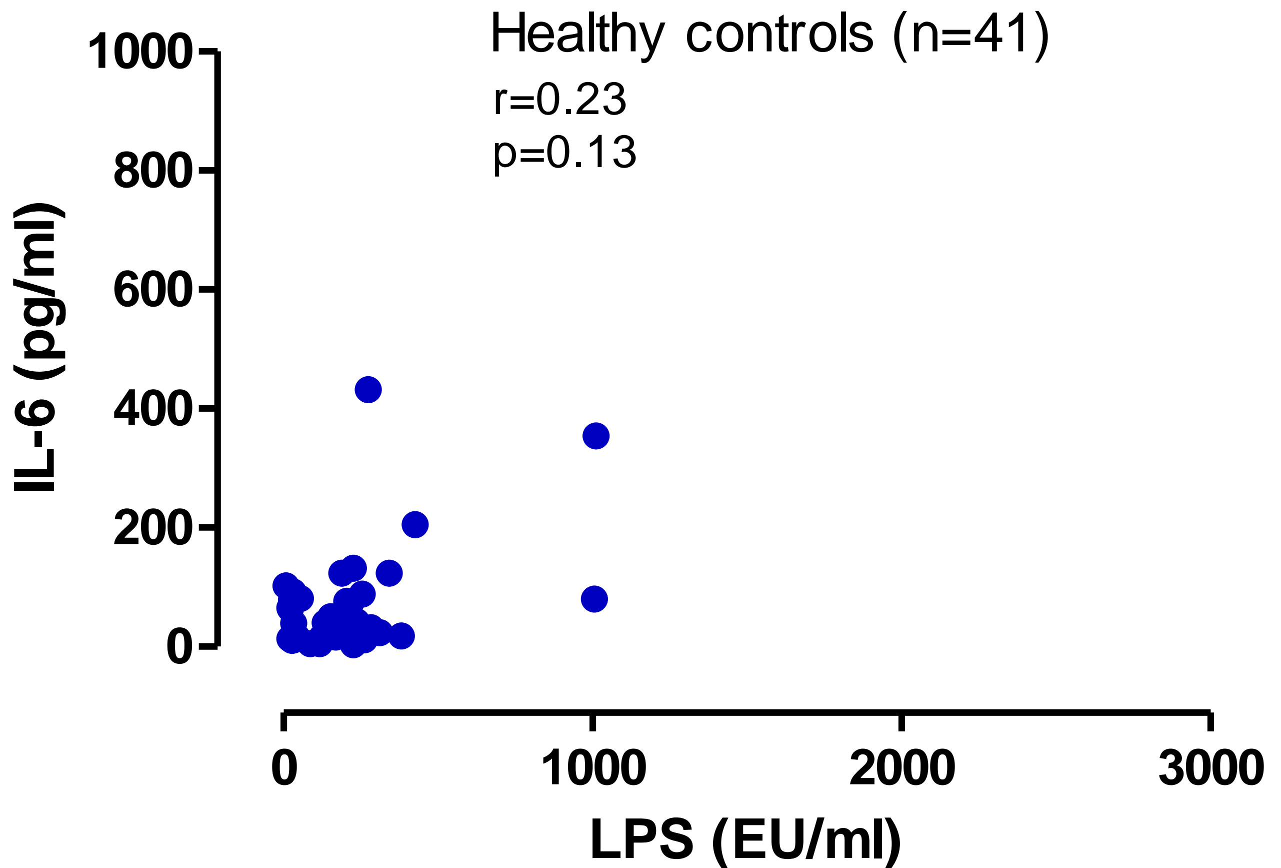


Figure 4.24: shows correlation between IL-6 and lipopolysaccharides in the healthy controls. There was positive but non-significant correlation in the healthy controls. Spearman's correlation is shown.

4.8.4: Correlation between IL-6 and 16S rRNA copy number in participants with EE

In participants with EE, there was positive and significant correlation between IL-6 and 16S rRNA copy number (r= 0. 47; p<0.001) as shown (Figure 4.25).

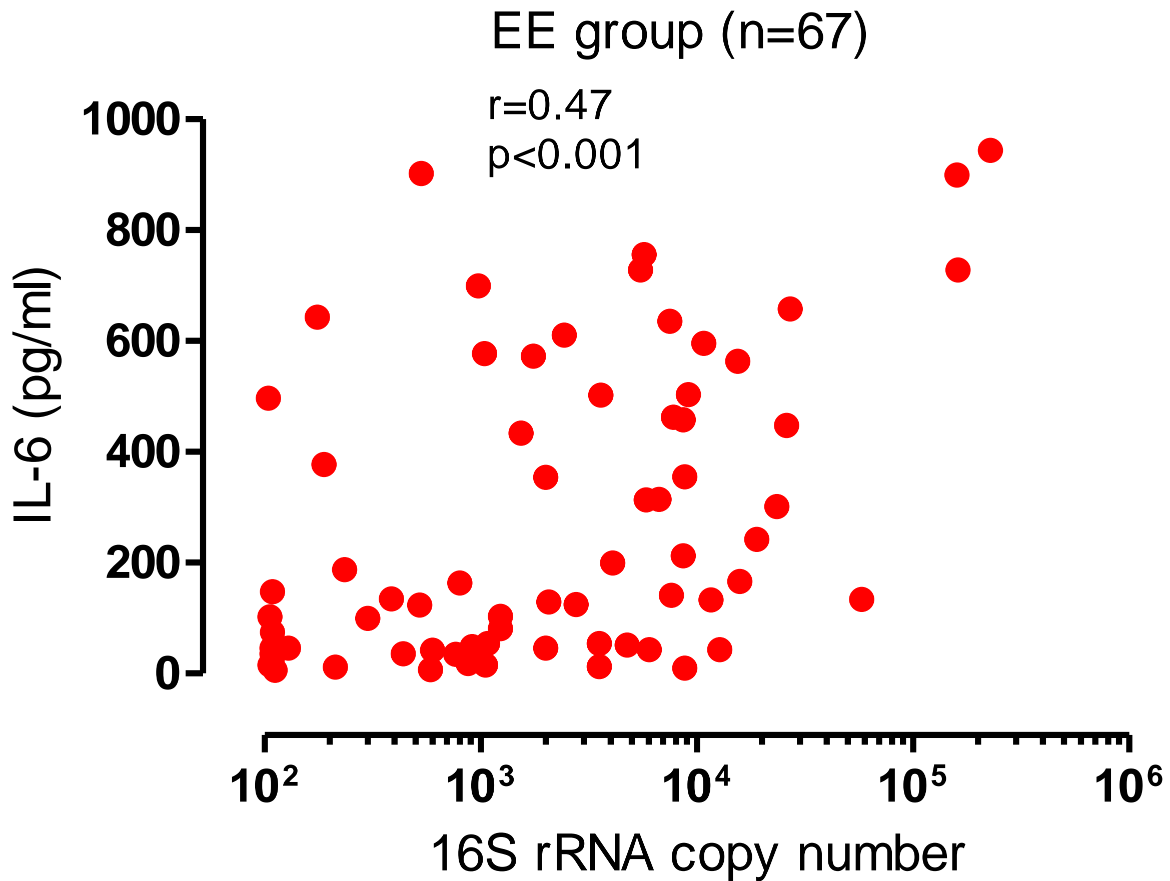


Figure 4.25: Shows correlation between IL-6 and 16S rRNA copy number in the environmental enteropathy. There was positive and significant correlation. Spearman's correlation is shown.

4.8.5: Correlation between IL-6 and 16S rRNA copy number in participants with HSS

In participants HSS, there was positive and significant correlation between IL-6 and 16S rRNA copy number (r= 0. 39; p<0.003) as shown (Figure 4.26).

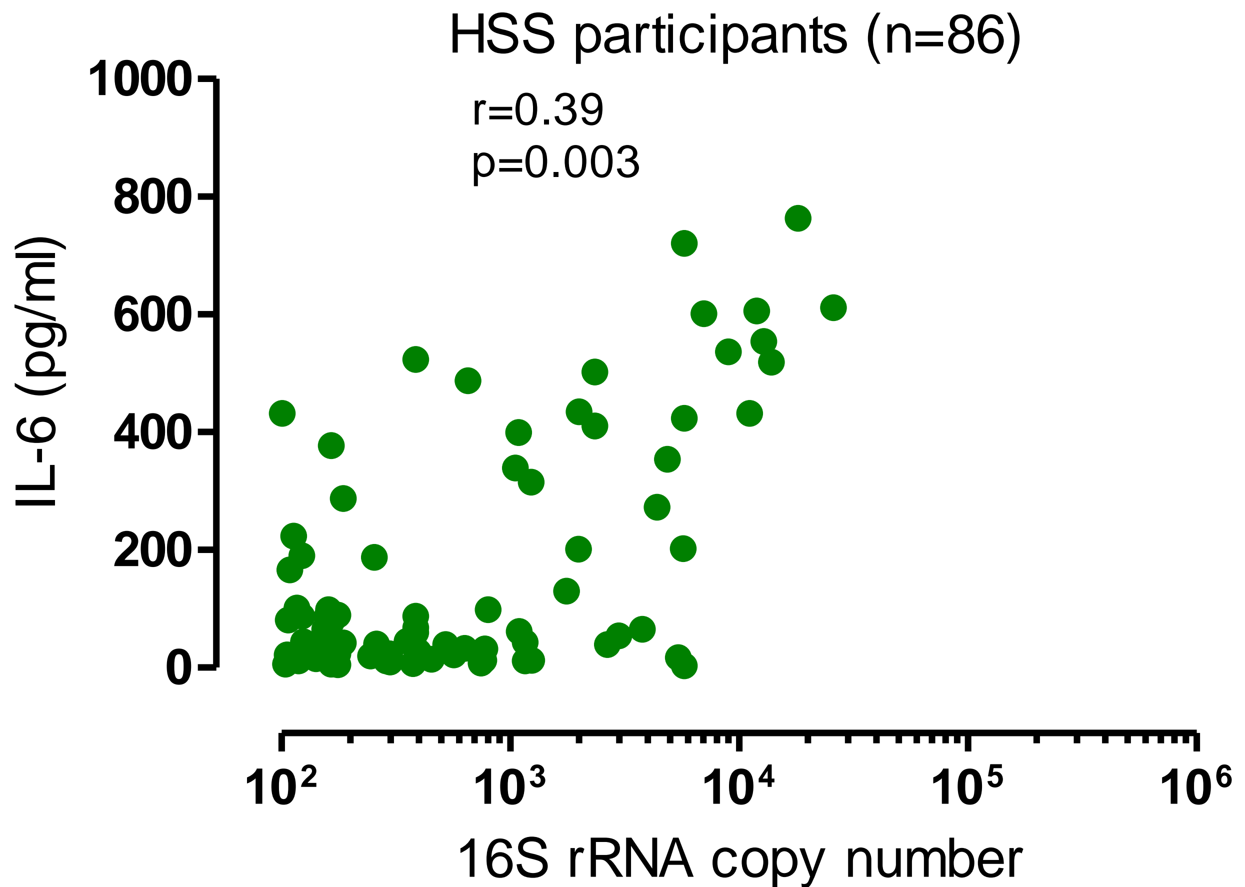


Figure 4.26: Correlation between IL-6 and 16S rRNA copy number in participants with hepatosplenic shistosomiasis. There was positive and significant correlation. Spearman's correlation is shown.

4.8.6: Correlation between IL-6 and 16S rRNA copy number in Healthy Controls

In healthy controls, there was positive and significant correlation between IL-6 and 16S rRNA copy number (r= 0. 33; p=0.06) as shown (Figure 4.27).

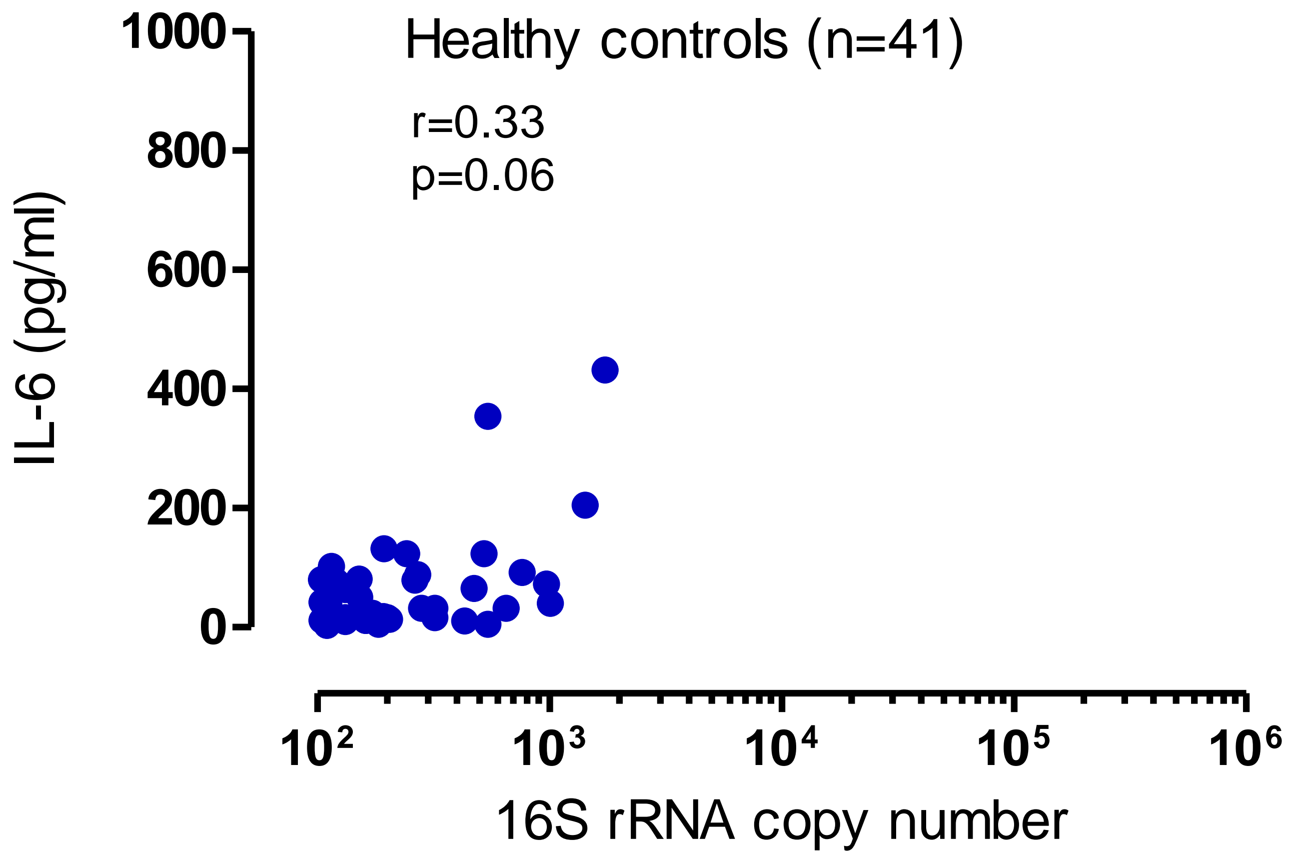


Figure 4.27: Shows correlation between IL-6 and 16S rRNA copy number in healthy controls. There was positive but non-significant correlation. Spearman's correlation is shown.

4.8.7: Correlation between IL-6 and TLRLs in participants with EE

In participants with EE, there was positive and significant correlation between IL-6 and TLRLs (r= 0. 66; p<0.001) as shown (Figure 4.28).

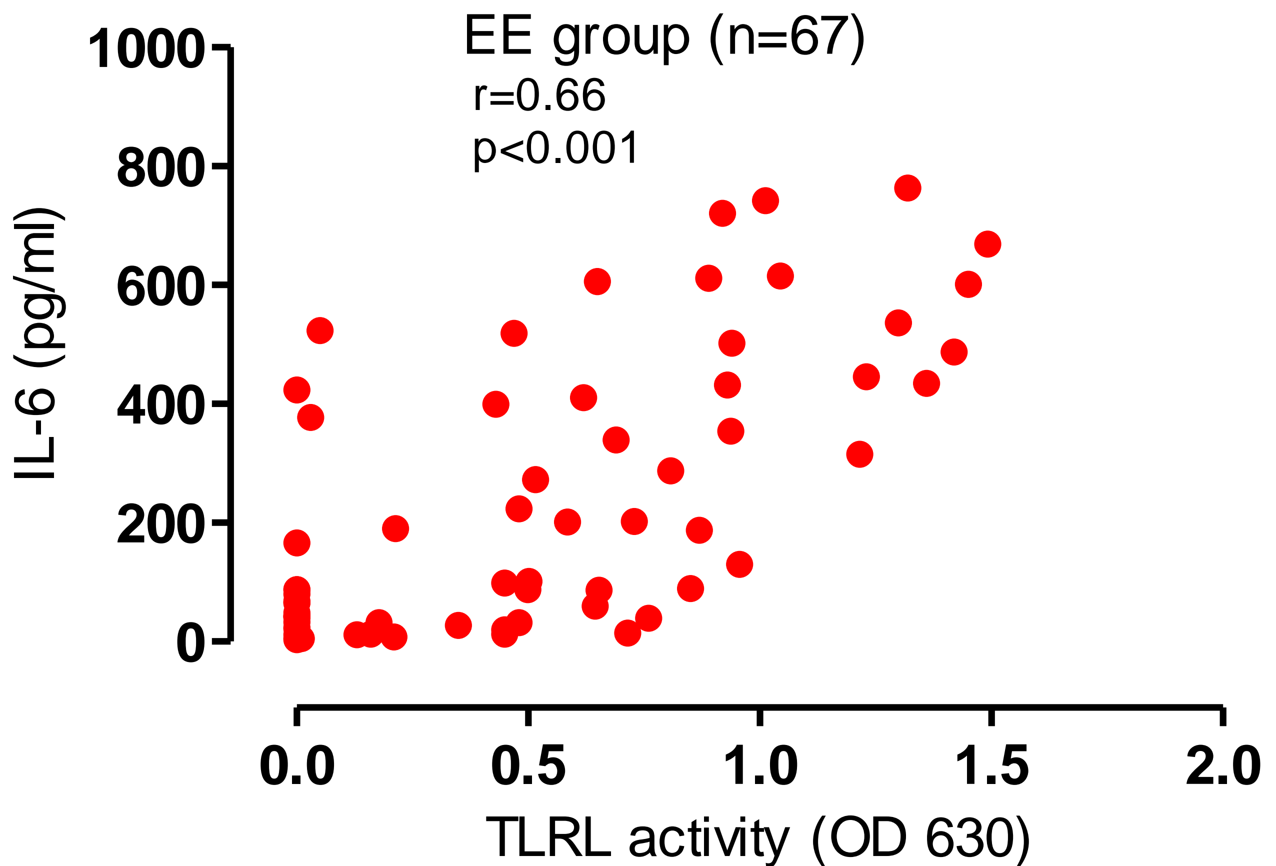


Figure 4.28: Shows correlation between IL-6 and Toll-like receptor ligands activity in participants with environmental enteropathy. There was positive and significant correlation between IL-6 and Toll-like receptor ligands activity. Spearman's correlation is shown.

4.8.8: Correlation between IL-6 and TLRLs in participants with HSS

There was positive and significant correlation between IL-6 and TLRLs activity in participants with HSS (r=0.53, p<0.001) as shown (Figure 4.28).

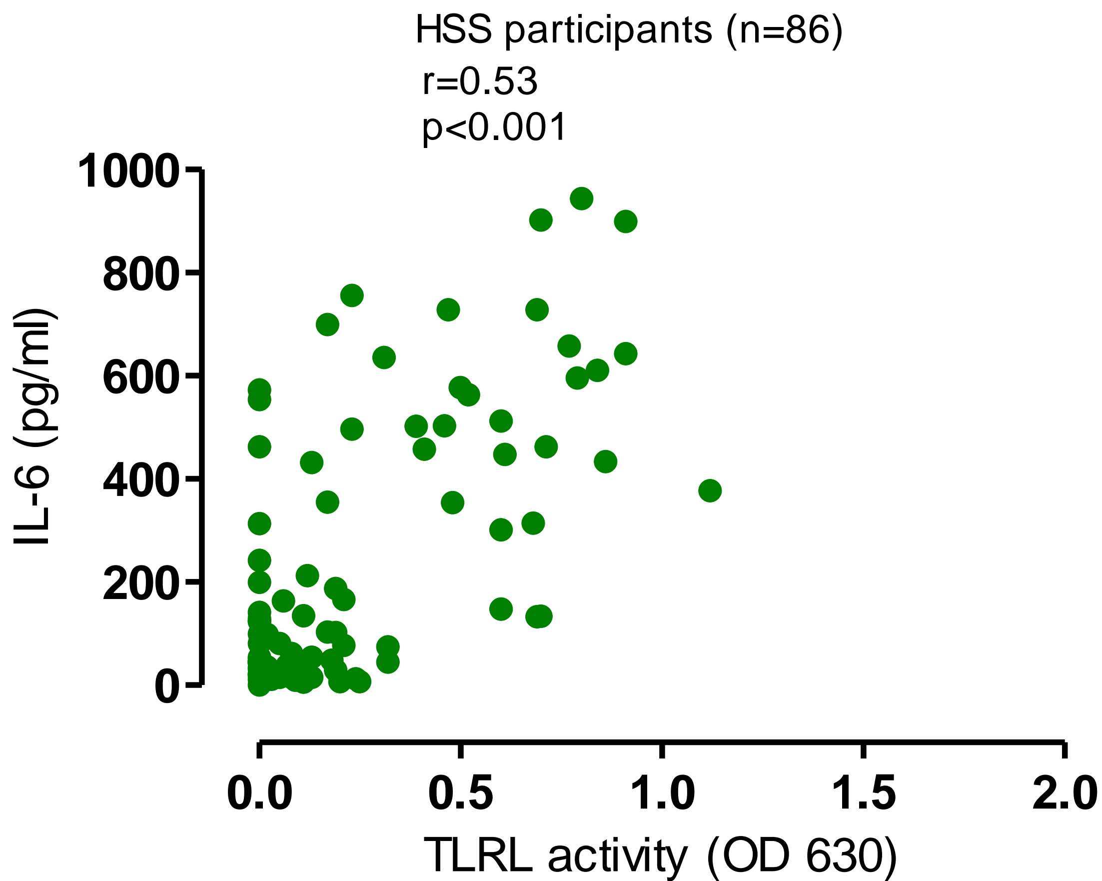


Figure 4.29: Correlation between IL-6 and Toll-like receptors activity in participants with hepatosplenic schistosomiasis. There was positive and significant correlation between IL-6 and Toll-like receptor ligands activity. Spearman's correlation is shown.

4.8.9: Correlation between IL-6 and TLRLs activity in Health Controls

There was positive and significant correlation between IL-6 and TLRLs activity in healthy controls (r=22, p=0.21) as shown (Figure 4.29).

Figure 4.30: Shows correlation between IL-6 and Toll-like receptors activity in healthy controls. There was positive but non-significant correlation between IL-6 and Toll-like receptors activity. Spearman's correlation is shown.

4.9: Correlation between IL-10 and Direct Biomarkers of Microbial Translocation

4.9.1: Correlation between IL-10 and LPS in participants with EE

There was positive and significant correlation between IL-10 and LPS in participants with EE (r=0.36, p=0.003) as shown (Figure 4.29).

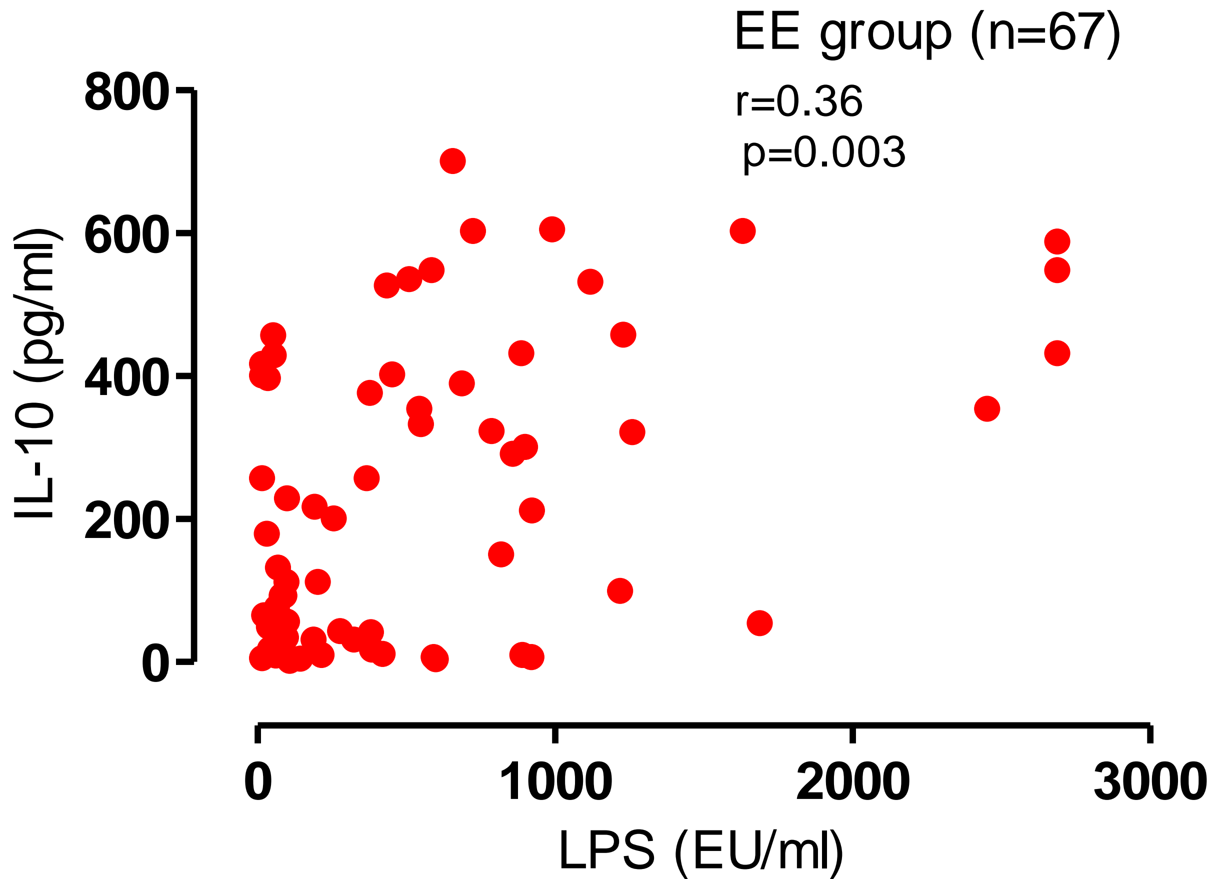


Figure 4.31: Correlation between IL-10 and LPS in participants with environmental enteropathy. There significant positive and significant correlation between IL-10 and LPS. Spearman correlation is shown.

4.9.2: Correlation between IL-10 and LPS in participants with HSS

There was positive and significant correlation between IL-10 and LPS in participants with HSS (r=0.28, p=0.008) as shown (Figure 4.30).

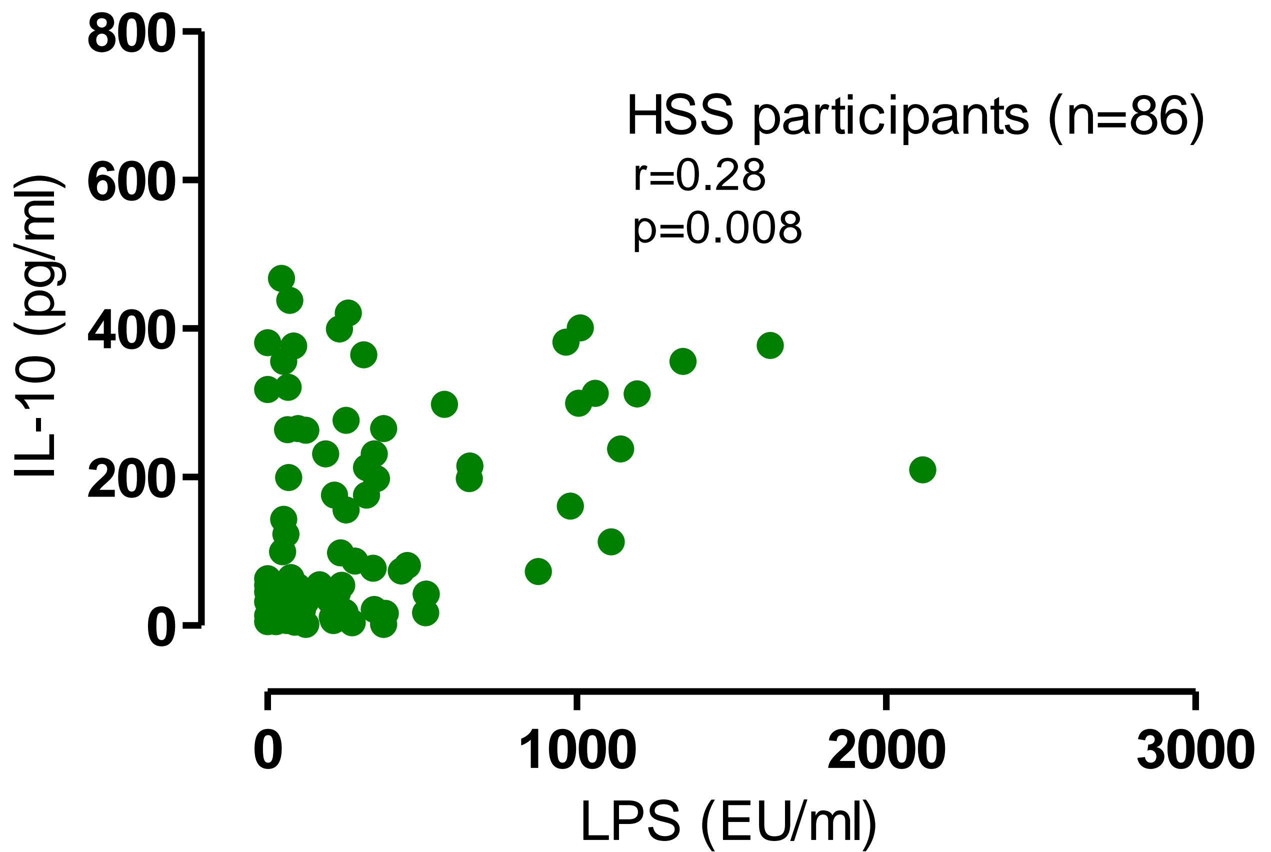


Figure 4.32: Shows correlation between IL-10 and LPS in participants with hepatosplenic shistosomiasis. There was positive and significant correlation between IL-10 and LPS. Spearman correlation is shown.

4.9.3: Correlation between IL-10 and LPS in Healthy Controls

There was positive and significant correlation between IL-10 and LPS in health controls (r=0.21, p=0.10) as shown (Figure 4.31).

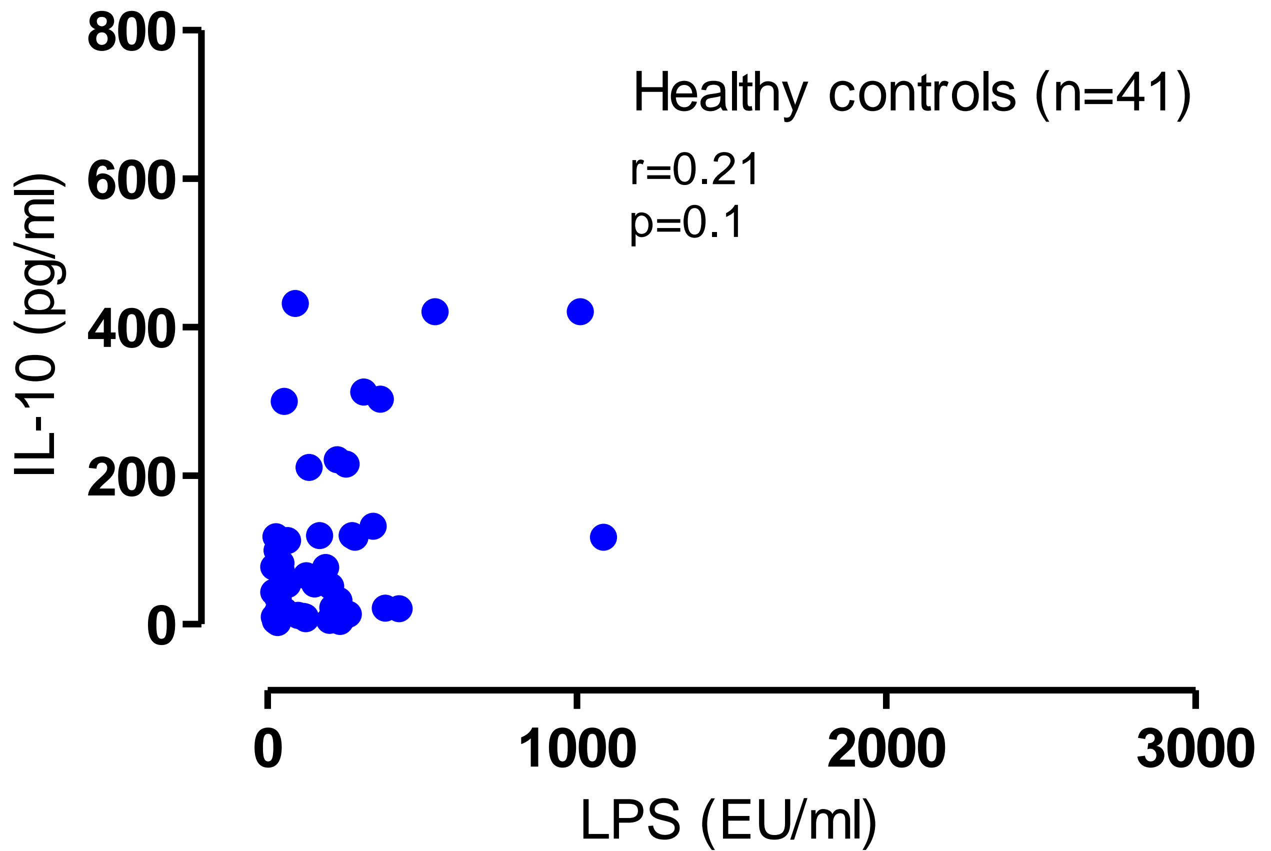


Figure 4.33: Shows correlation between IL-10 and LPS in healthy controls. There was positive but non-significant correlation between IL-10 and LPS. Spearman correlation is shown.

4.9.4: Correlation between IL-10 and 16S rRNA copy number in participants with EE

There was positive and significant correlation between IL-10 and 16S rRNA copy number in participants with EE (r=0.38, p=0.002) as shown (Figure 4.32).

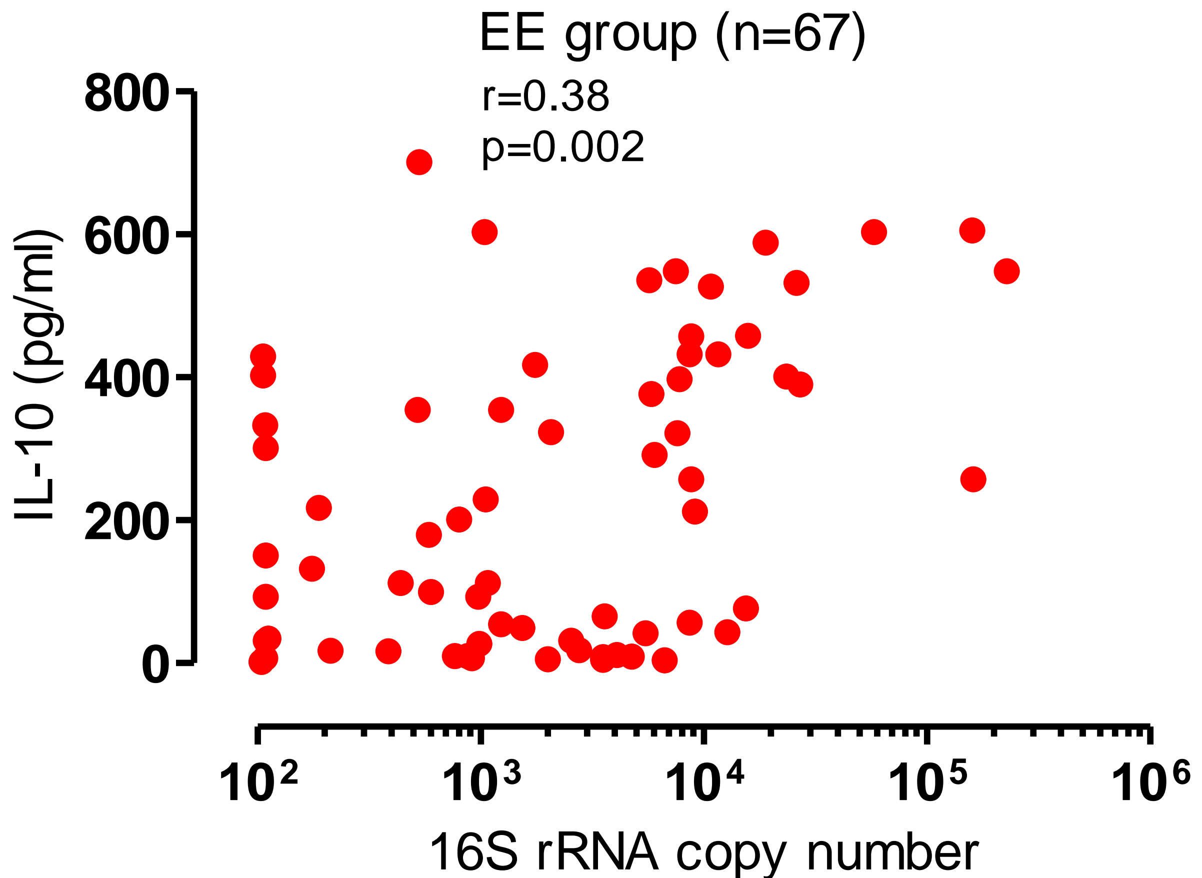


Figure 4.34: Shows correlation between IL-10 with 16S rRNA copy number in participants with environmental enteropathy. There was positive and significant correlation between IL-10 and 16S rRNA copy number. Spearman's correlation is shown.

4.9.5: Correlation between IL-10 and 16S rRNA copy number in participants with HSS

There was positive and significant correlation between IL-10 and 16S rRNA copy number in participants with HSS (r=0.38, p=0.004) as shown (Figure 4.32).

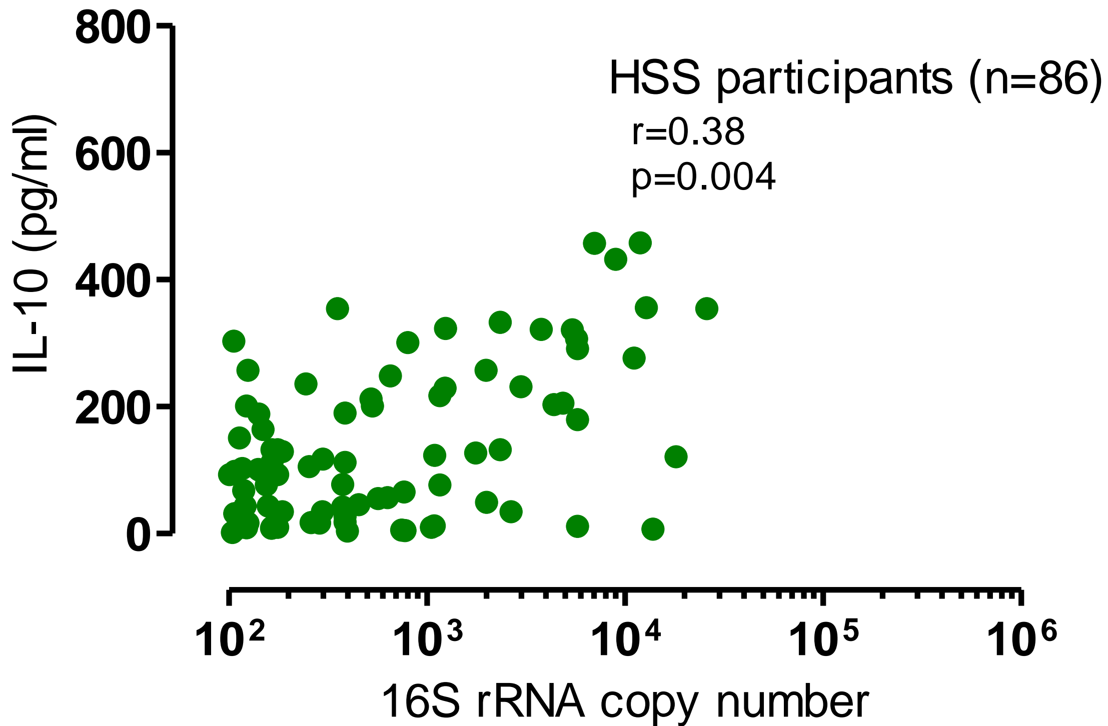


Figure 4.35: Correlation between IL-10 with 16S rRNA copy number in participants with hepatosplenic shistosomiasis. There was positive and significant correlation between IL-10 and 16S rRNA copy number. Spearman's correlation is shown.

4.9.6: Correlation between IL-10 and 16S rRNA copy number in Healthy Controls

There was positive and significant correlation between IL-10 and 16S rRNA copy number in healthy controls (r=0.24, p=0.09) as shown (Figure 4.33).

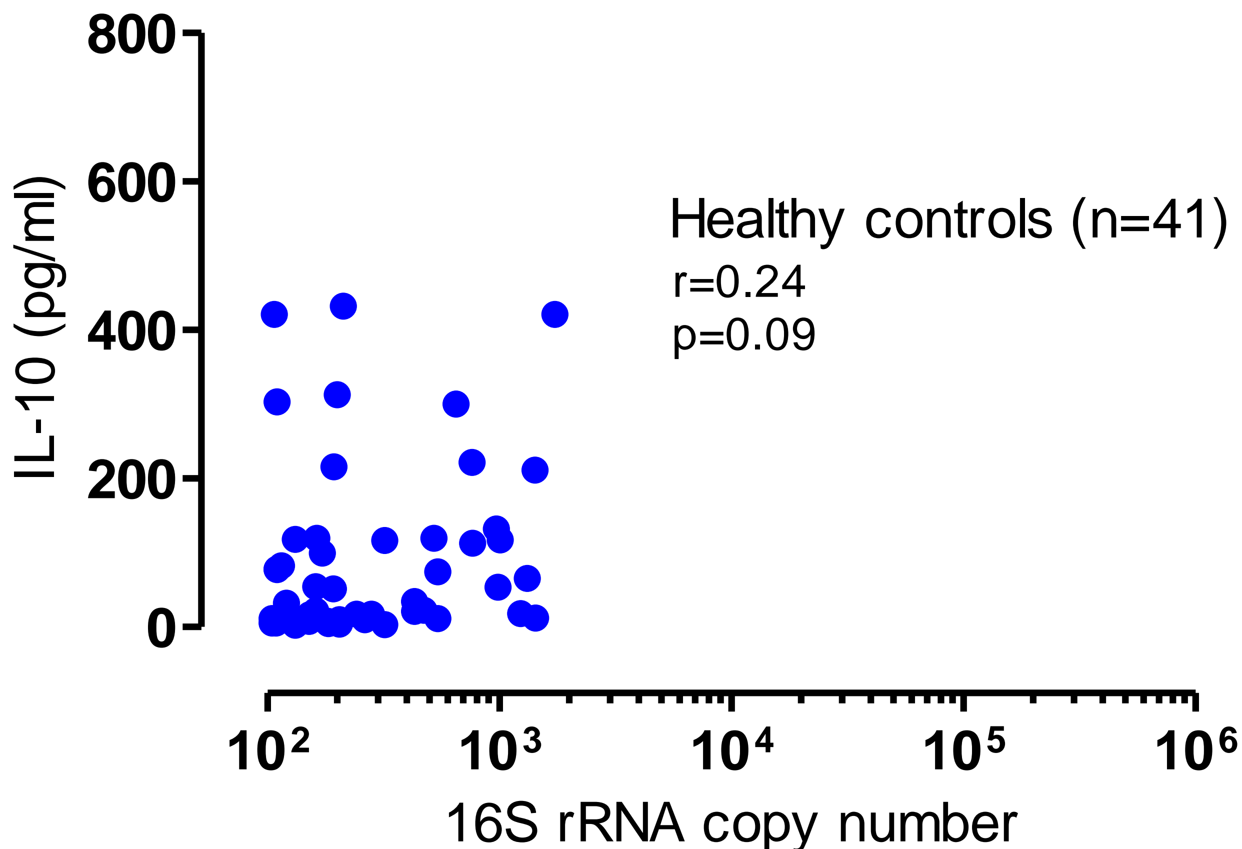


Figure 4.36: Shows correlation between IL-10 with 16S rRNA copy number in healthy controls. There positive but non-significant correlation between IL-10 and 16S rRNA copy number. Spearman's correlation is shown.

4.9.7: Correlation between IL-10 and TLRLs activity in participants with EE

There was positive and significant correlation between IL-10 and Toll-like receptors activity in participants with EE ($r=0.58$, $p<0.001$) as shown (Figure 4.34).

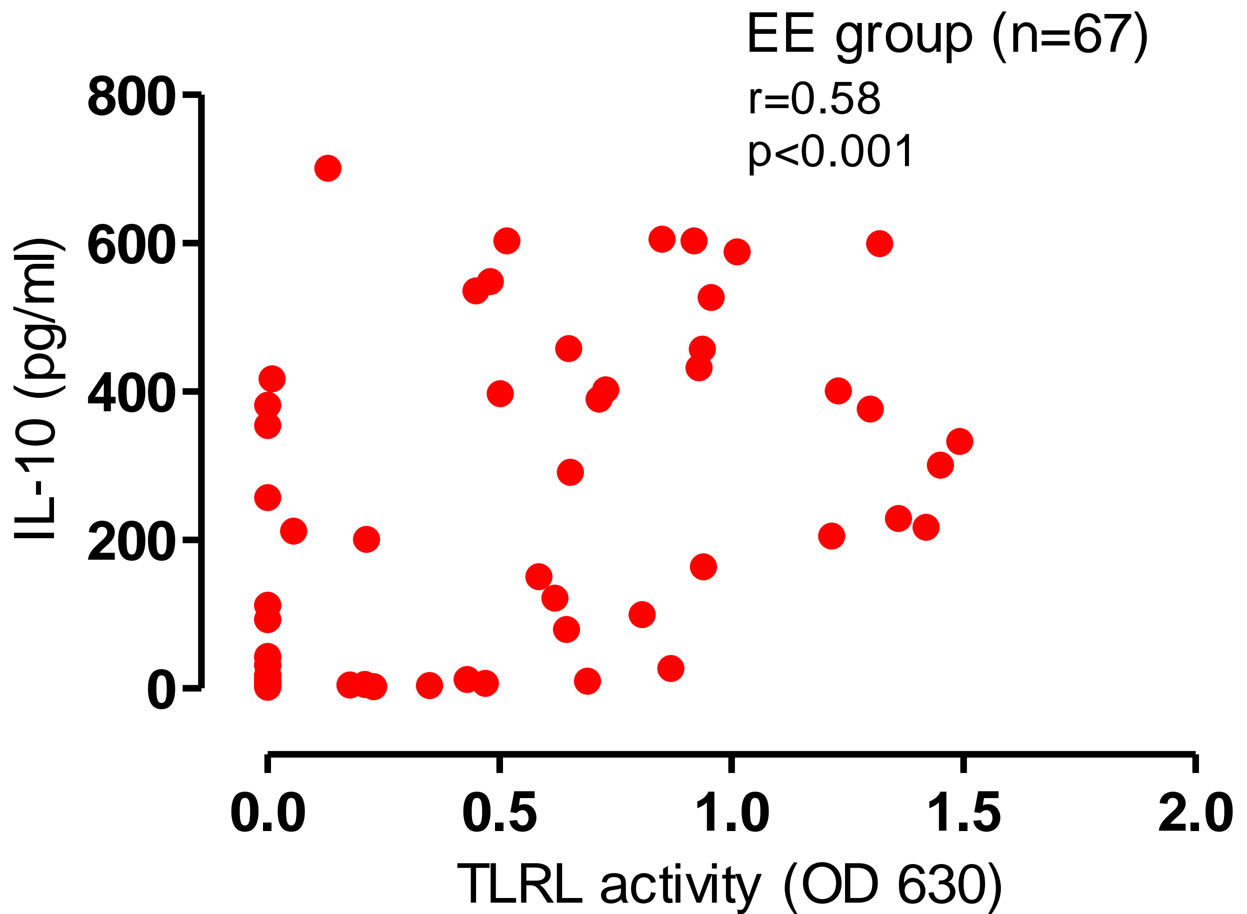


Figure 4.37: Correlation between IL-10 and Toll-like receptors activity in participants with environmental enteropathy. There was positive and significant correlation between IL-10 and Toll-like receptors activity. Spearman's correlation is shown.

4.9.8: Correlation between IL-10 and TLRLs activity in participants with HSS

There was positive and significant correlation between IL-10 and TLRLs activity in participants with HSS (r= 0.32, p=0.004) as shown (Figure 4.35).

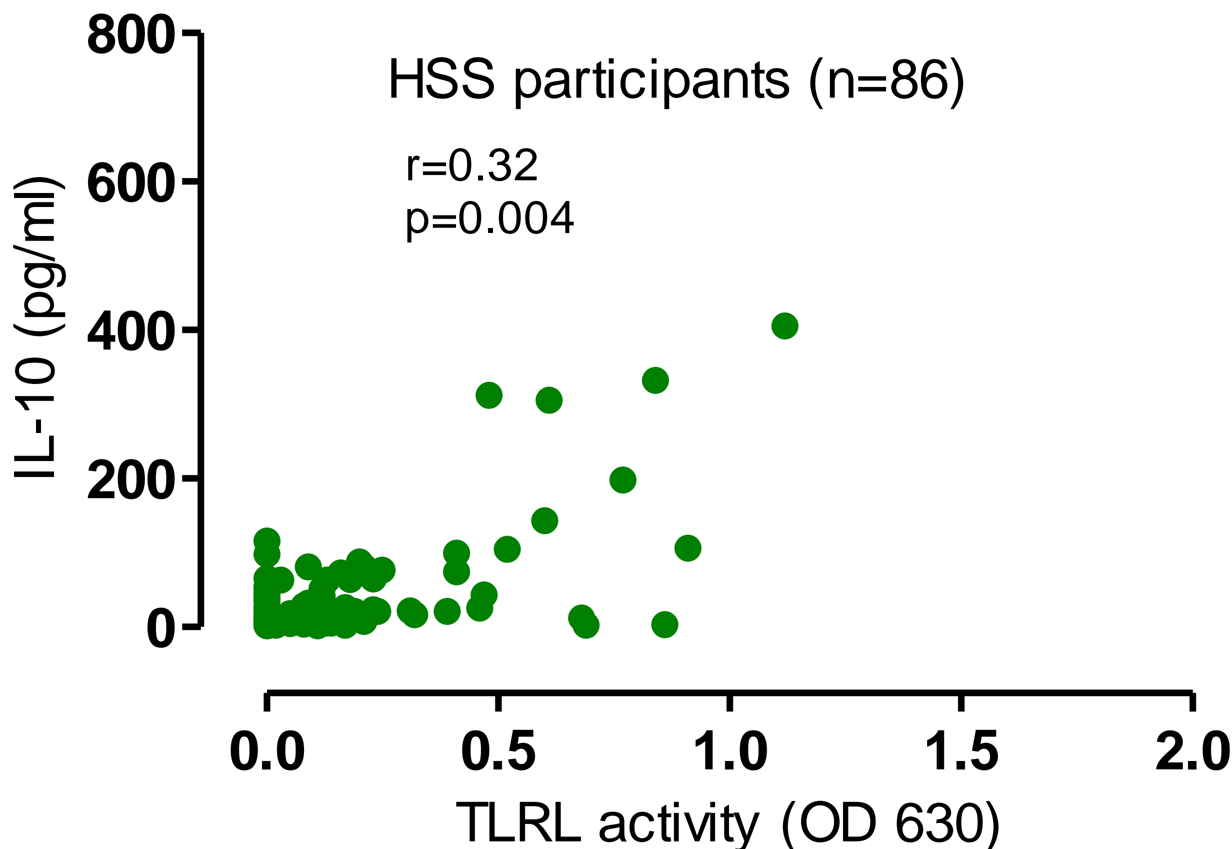


Figure 4.38: Shows correlation between IL-10 and Toll-like receptors in participants with hepatosplenic shistosomiasis. There was positive and significant correlation between IL-10 and Toll-like receptors activity. Spearman's correlation is shown.

4.9.9: Correlation between IL-10 and TLRLs activity in Healthy Controls

There was positive but non-significant correlation between IL-10 and TLRLs activity in healthy controls (r=0.25, p=0.09) as shown (Figure 4.36).

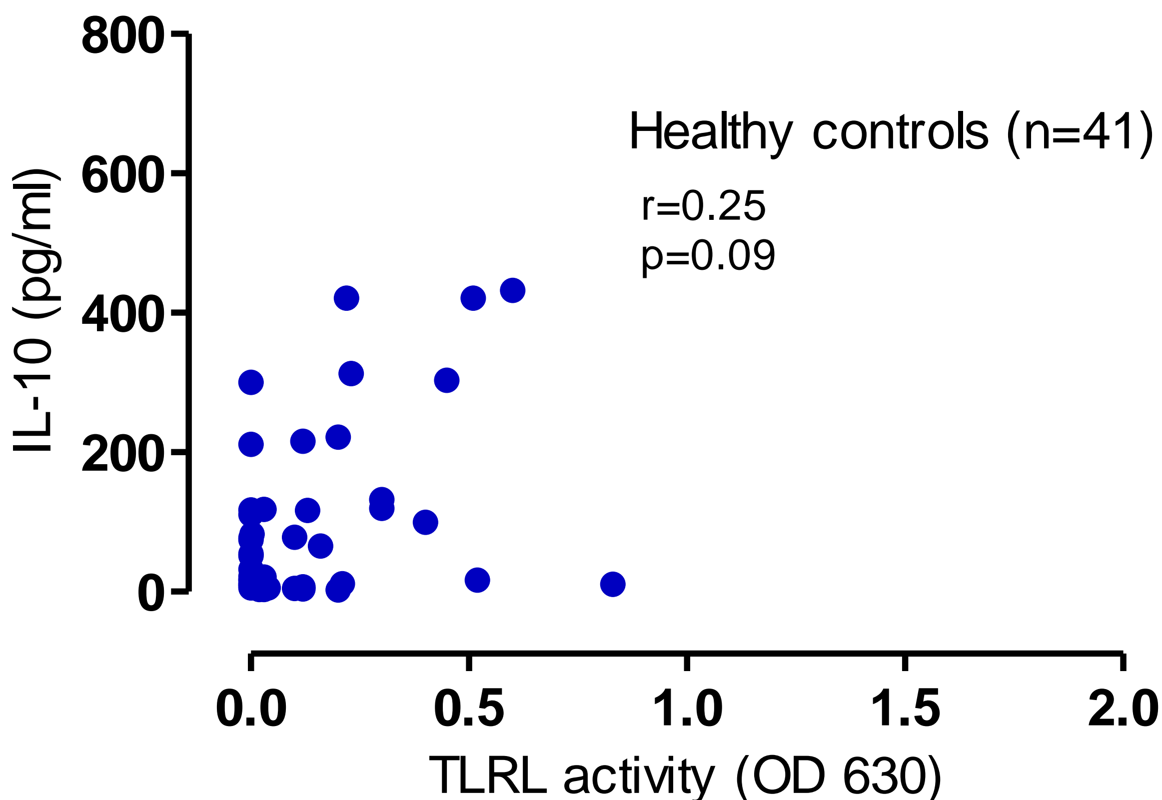


Figure 4.39: Shows correlation between IL-10 and Toll-like receptors activity in healthy controls. There was positive but non-significant correlation between IL-10 and Toll-like receptors activity. Spearman's correlation is shown.

4.10 Multivariate Multiple Regression Model

In the final analysis to rule out confounders and to obtain a summary of the relationship between direct biomarkers and immune activation, multivariate multiple regression models were constructed taking LPS, 16S rRNA copy number and TLRLs as independent variables while cytokines (TNF-α, IL-6, IL-10, IL-4 and IL-2) as dependent variables and controlling for baseline differences (age, haemoglobin, Platelet, WBC and HIV). Stepwise backward Multivariate multiple regression analyses were conducted to examine the relationship between direct biomarkers of microbial translocation (LPS, 16S rRNA copy number and TLRLs) and immune activation (TNF-

α, IL-6, IL-10, IL-4 and IL-2) with the probability of removal set at 20%, in the EE group, a good model fit was obtained ($R^2 = 0.526$, $F = 47.53$, p<0.001) which significantly predicted TNF-α, IL-6 and IL-10. In the HSS group a less impressive but still significant fit was obtained ($R^2 = 0.382$, $F = 22.43$, p=0.002). Of note in this model LPS did not statistically predict activation of IL-6 and IL-10. In healthy controls no satisfactory model was obtained ($R^2 = 0.040$, $F = 1.03$, $p = 0.38$. IL-4 and IL-2 were not predicted by any direct biomarker in the models. Akaike Information Criteria and Bayesian Information Criteria were used to test for model fit. After regression heteroscedasticity was tested using Breusch-Pagan test using *hettest* command in STATA. Where significant it is indicated by asterisk as shown in Table 4.6.

Table 4.6: Multivariate multiple regression analysis of relationship between cytokines and direct biomarkers of microbial translocation

	EE (n=67)		HSS participants (n=86)		Healthy controls (n=41)	
	B	95% CI	*B*	95% CI	*B*	95% CI
TNF-α						
LPS	0.04	0.03 to 0.14*	0.06	0.03 to 0.17*	0.02	-0.09 to 0.13
16S rRNA	0.004	0.002 to 0.007*	0.006	0.001 to 0.01*	0.003	-0.007 to 0.01
TLRLs	11.3	6.6 to 15.4**	6.1	1.76 to13.9*	3.6	-0.95 to 8.45
IL-6						
LPS	0.006	0.01 to 0.09*	0.09	-0.008 to 0.17	0.02	-0.02 to 0.15
16S rRNA	0.04	0.0009 to 0.008*	0.01	0.002 to 0.02*	0.007	-0.001 to 0.02
TLRLs	7.7	2.11 to 9.98**	4.5	1.1 to 12.3*	1.4	-0.5 to 17.3
IL-10						
LPS	0.04	0.009 to 0.71*	0.03	-0.008 to 0.06	0.02	-0.011 to 0.05
16S rRNA	0.004	0.004 to 0.02*	0.002	0.0009 to 0.008*	-0.002	-0.003 to 0.004
TLRL	1.96	0.95 to 4.9*	1.4	1.1 to 3.52*	0.64	-2.2 to 3.5
IL-4						
LPS	-0.003	-0.02 to 0.01	0.001	-0.002 to 0.003	0.0002	-0.0003 to 0.0007
16S rRNA	0.002	-0.003 to 0.008	-0.001	-0.002 to 0.001	-0.0001	-0.0003 to 0.0006
TLRLs	1.2	-1.6 to 4.1	-3.1	-9.5 to 3.2	-0.08	-0.22 to 0.07
IL-2						
LPS	-0.001	-0.007 to 0.005	-0.002	-0.01 to 0.007	0.002	-0.01 to 0.008
16S rRNA	-0.005	-0.01 to 0.01	-0.006	-0.007 to 0.0009	-0.0001	-0.008 to 0.001
TLRLs	0.46	-0.75 to 1.67	-1.5	-4.1 to 3.7	-0.02	-0.1 to 0.08

Data are given as regression coefficient (B) and 95% confidence interval. R^2 is 0.526 for the EE model, 0.382 for the HSS model, and 0.040 for the healthy controls model. Outcome variables were log-transformed and reported back on the original scale of measurement.

****p<0.05 significant values different from reference category adjusted for all explanatory variables in the model, age, hemoglobin, Platelet, WBC and HIV.***

*****p<0.01 significant values different from reference category adjusted for all explanatory variables in the model, age, hemoglobin, Platelet, WBC and HIV.***

LPS: Lipopolysaccharide; 16S rRNA gene: 16S ribosomal RNA gene; TLRL: Toll-Like receptor ligand; TNF-α: tumor necrosis factor-alpha; IL-6: interlukin-6; IL-10: interlukin-10; IL-4: interlukin-4; IL-2:interlukin-2

CHAPTER FIVE: DISCUSSION

5.1 Overview of the Findings

This study investigated direct biomarkers of MT as predictors of immune activation (cytokines) in participants with EE compared with participants with HSS or healthy controls. The direct biomarkers investigated were LPS, 16S rRNA copy number and TLRLs measured using activity in the RAW-Blue cell line. The study also investigated cytokines produced as a result of immune responses to PAMPs in cell culture supernatant. Plasma cytokines were not measured because the interest of the study was to demonstrate in vitro model of immune activation due to microbial products that could be are present in blood of the EE and HSS participants. Plasma biomarkers of host response to MT were investigated. These were CRP, sCD14 and sCD163 and LBP. The study also analysed correlations between LPS, 16S rRNA copy number, TLRLs and cytokines also with plasma biomarkers of host response to MT. In addition, the study sought to investigate and determine which direct biomarker could best predict immune activation in individuals with EE or HSS participants. At baseline, some demographic characteristics were different among the three groups of participants. EE is known to be prevalent in both children and adults (Crane *et al*., 2015). Most studies however, have been conducted in children (Campbell *et al*., 2003; Mannary *et al.,* 2014, Yu *et al*., 2016) and only few have been conducted in adults (Loius-Auguste *et al.,* 2014; Kelly *et al.,* 2016). This could probably be to its far greater consequences in children than in adults. One of the determining factors for development of EE is repeated exposure to fecal-oral contamination (Salazar-Lindo *et al.,* 2004; Keusch *et al.,* 2013). Cumulative amounts of environmental contamination leading to the EE outcome overtime have been described by the term

exposome (Miller and Jones, 2014). Suggestions have been made to assess the role of repeated environmental exposure to microbial contaminants and the time taken for EE to develop in young children as one way of attempting to answer the question of causality of EE (Mapesa *et al*., 2015).

In this study participants with were all from an urban area with poor sanitation and hygiene. Participants with HSS and healthy controls were recruited from the University Teaching Hospital, Lusaka, Zambia so the information about their residences was not the same. There were no difference in sex distribution that was observed among EE or HSS similar to what has been reported elsewhere (Kelly *et al*., 2016) and (Sinkala *et al*., 2016) respectively. Participants with HSS were more likely to be older compared to participants with EE or healthy controls. This was similar to other findings which suggested that HSS becomes more of a chronic disease as one gets older and is more likely to be acquired with increase in age (Mutengo *et al*., 2014). This study recruited more women in participants with EE (57%) similar to previous studies in the same area (Kelly *et al*., 2010; Kelly *et al*., 2015) in agreement what others have suggested that given equal chance, women are more likely than men to participate in research studies (Curtin *et al*., 2000; Moore and Tarnai, 2002; Zulu *et al*., 2014). In participants with HSS and healthy controls, the numbers of women were not statistically different 52% and 49% respectively. In this study about 22% of individuals with EE were HIV sero-positive and some studies have reported that severity of enteropathy in HIV infected adults is almost indistinguishable (with the exception of crypt depth) from that of uninfected adults with EE (Prendergast and Kelly, 2012). Permeability (measured by lactulose:rhamnose ratio) is increased only in AIDS, not in early HIV infection (Kelly *et al*, 2004). Those with cryptosporidiosis and other related opportunistic infections were excluded by stool examination in all participants, and additionally, those who had reported cases

of diarrhoea two weeks prior to the commencement of the study were excluded as per one of the exclusion criteria. A previous study found that in adults with EE, plasma LPS concentrations did not differ in HIV sero-negative compared to HIV sero-positive individuals (Kelly *et al*., 2016). Therefore, in this study HIV infection is unlikely to explain the difference in the biomarkers of MT.

There is increasing indication that MT leads to immune activation in many different disorders associated with compromised intestinal barrier such as EE (Kelly *et al*., 2016) and HSS (Sinkala *et al*., 2016). MT is difficult to measure and some studies have suggested positive bacterial cultures of mesenteric lymph nodes (MLNs) as a direct method of measuring the phenomenon (Berg *et al*., 1995) which has a number of limitations. A number of studies have suggested the use of biomarkers of microbial origin such LPS and bacterial DNA (16S rRNA genes) as effective direct biomarkers of MT (Marchetti *et al*., 2013; Bellot *et al*., 2013; Koutsonas *et al*., 2015). This study compared three different approaches to measurement of MT in blood samples by measuring LPS, 16S rRNA and Toll-like receptor ligands (TLRLs) activity which detects all possible PAMPs in plasma. The question of whether plasma direct biomarkers of MT correlate with host response to MT and immune activation in EE and HSS participants was also addressed.

5.2 Microbial Translocation and Lipopolysaccharide

Lipopolysaccharide (LPS), a heat stable endotoxin with a molecular weight of 10-20kDa present only in Gram-negative bacteria, is the major cause of septic shock in humans. Its lipid A moiety component is responsible for the stimulation of strong immune response (D'Ettorre *et al*., 2011; Sandler and Douek, 2012). LPS has been widely used as a direct biomarker of MT in many

disorders associated with compromised gut barriers (Marchetti *et al*., 2013). This study found LPS was significantly higher in the EE group compared with HSS patients or healthy controls. Surprisingly, there was no significant difference in LPS levels between HSS patients and healthy controls. A study of transcriptomic and endoscopic analysis of adult Zambians with EE, reported elevated levels of plasma LPS (Kelly *et al*., 2016) suggesting the occurrence of MT. In inflammatory bowel disease, Crohn's disease, patients had significantly higher plasma LPS compared with healthy controls (Pastor Rojo *et al*., 2007) indicating the occurrence of MT. Similarly, in patients with ulcerative colitis plasma LPS was reported significantly elevated compared with healthy controls (Pasternak *et al*., 2010). In both these IBDs the intestinal histological features were reported to be similar to EE (Korpe and Petri, 2012; Keusch *et al*., 2014) suggesting the occurrence of MT due to compromised intestinal barrier function. In HIV/AIDS infection it has been reported that patients develop HIV enteropathy due to significant depletion of CD4+ T-cells in the intestinal mucosa leading to compromised intestinal barrier resulting into MT. HIV-positive patients were reported to have significantly elevated plasma LPS compared with HIV-negative individuals (Jiang *et al*., 2009; Cassol *et al*., 2010). In a cross-sectional study conducted in individuals with EE, however, no difference in plasma LPS levels between those who were HIV-positve and HIV-negative was reported (Kelly *et al*., 2016). This suggested a possible masking effect that HIV infection could potentially have on the severity of EE. In this study, there was no difference in direct biomarkers of MT between HIV-positive and HIV-negative individuals in the EE group. This was the case even after adjustment for HIV status in multivariate multiple regression model, where the levels of significance were observed not to change suggesting that HIV had no effect on the severity of EE.

In HSS, one of the major consequences of the disease is portal hypertension which is associated with oesophageal varices, gastric varices or both. Variceal bleeds which result from increased portal pressure may lead to MT (Sinkala *et al*., 2016). A similar mechanism is hypothesized in cirrhosis in which a study showed that cirrhotic patients had significantly elevated plasma LPS compared with healthy controls (Vlachogiannakos *et al*., 2013) suggesting MT due to oesophageal or gastric varices. On the contrary, in this study plasma LPS levels were found not to be different between participants with HSS compared to healthy controls in agreement with the findings of Sinkala and colleagues (2016). This could be explained by what others have reported that some bacteria especially members of the Gram-negative bacteria are capable of crossing normal histological intestinal epithelium (Weist *et al*., 2013). It has also been hypothesized that exposure to LPS through burning of animal dung used as cooking fuel could cause systemic endotoxin contamination via lungs or gut. Animal dung is known to contain bacterial endotoxins as well as other cellular components of microbial origin (Semple *et al*., 2010; Bauer *et al*., 2011). In this study the use of dung as fuel in the participants was not assessed. It has also been suggested that due to the short half-life of LPS it is possible to miss some transient incidences of bacteremia when LPS is used as a biomarker of MT (Albiloos *et al.,* 2003).

Translocation of LPS activates TLR4 expressed by most cells of the innate immune system such as macrophages causing the release of pro-inflammatory cytokines. Studies have linked the severity of cirrhosis to the number of microbes and their translocation across the intestinal barrier. Comparisons have been made between patients with cirrhosis and healthy controls and results suggest that in cirrhotic patients there was direct correlation between disease and number of pathogenic bacteria and patients were observed to have less of normal flora than healthy controls (Chen *et al*., 2011). In this study, there was positive and significant correction between LPS and

sCD14 or LPB in participants with EE while in participants with HSS the significant correlation was only with LBP suggesting host response to MT. There was no significant correlation in the healthy controls. Overall, combining the participants with EE and HSS stronger correlations were obtained between LPS and all biomarkers of host response to MT suggesting that when the two conditions co-exist, a much greater effect may be observed. In patients with cirrhosis, some studies have reported that LPS aggravate portal hypertension and it is known to be an important driver in the development of small bowel mucosal changes (Reibeiger *et al*., 2013). LPS has also been known to raise intrahepatic resistance. The reduction of plasma LPS also has been shown to correlate with reduction in portal venous pressure gradient (Meng *et al*., 2016). Further, in cirrhotic patients apart from LBP and pro-inflammatory cytokines, some surrogate biomarkers of MT such as sCD14 and sCD163 have been shown to be significantly elevated compared with healthy controls (Guerra-Ruiz *et al*., 2010) and have also been observed to be associated with severity of disease. In this study, the findings of significant elevated plasma LPS in participants with EE compared to those with HSS or healthy controls suggest that there was more MT in those with EE and the correlation with LPS could suggest the magnitude of translocation.

5.3 Microbial Translocation and 16S rRNA copy Number

The16S rRNA gene codes for ribosomal RNA component of the smaller ribosomal subunit, the 30S subunit of prokaryotic cell (Schluenzen *et al*., 2000). Since the 16S rRNA genes are only present in bacteria their presence in human blood may be considered as evidence for exposure to bacteria (Marchetti *et al*., 2013; Bellot *et al*., 2013). In the current study, plasma 16S rRNA copy numbers were found to be significantly higher in individuals with EE compared to those with HSS or healthy controls. In participants with HSS, plasma 16S rRNA copy number was also

significantly higher compared to healthy controls. In a study in Malawian children, using 16S rRNA sequence, children with EE had more and diverse bacteria compared to children without EE (Yu *et al*., 2015) suggesting more MT in children with EE. In USA, a cross-sectional study was conducted to compare plasma I6S rRNA copy number between HIV-positive treatment naïve patients and HIV-negative. HIV-positive treatment naïve patients had significantly higher copy number than HIV-negative individuals (Jiang *et al*., 2009) suggesting compromised intestinal barrier leading to MT in HIV-positive treatment naïve patients. The investigators also reported no detectable 16S rRNA copy number in blood of healthy controls. Further, there was correlation between biomarkers of MT and immune activation. Suggesting that the higher the biomarkers of MT the more the immune system is activated. Similarly, in this study, there was significant positive correlation between 16S rRNA and LBP in both the EE and HSS participants respectively. This suggested that there was host response to MT in the EE and HSS participants but not in healthy controls. Contrary to results from Jiang and colleagues (2009), this study found 16S rRNA copy number in the healthy controls and there was no difference in 16S rRNA copy number between those who were HIV-positive and HIV-negative in the EE group. The discrepancy could be due to difference in sensitivity of the assay that was used in this study. First, the PCR protocol that was used in this study was designed for high sensitivity. It used very close primers (F519 and R785) designed to increase fluorescence detection (Ferri *et al*., 2010). Second, in this study the absolute quantification performed does not target a single gene and also used a highly efficient DNA extraction kit (QIAGEN) designed to minimize differences in the concentration and quality of DNA among sample batches. These factors were not considered in the study conducted by Jiang and colleagues (2009).

In a different cross-sectional study, plasma 16S rRNA was reported to be significantly higher in HIV-positive immunologic-non-responders compared with HIV-positive responders to highly active anti-retroviral therapy (HAART) treatment and it was shown that plasma 16S rRNA was associated with LPS in immunologic-non-responders. The study concluded that the correlation was enhanced due to increased systemic translocation of microbial products (Marchetti *et al.*, 2008) in agreement with this study. In a longitudinal study, HIV-infected patients starting HAART with CD4 count of less than 200cells/µl and others with CD4 count ≥250 cells/µl, both were found with elevated plasma 16S rRNA but with no difference between groups. Plasma 16S rRNA revealed that many different microorganisms were present before and after treatment in both groups and LPS was also elevated (Merlini *et al*., 2011). In this study, the median CD4 count was 514 cells/µl of blood for those participants who were HIV-positive in participants with EE suggesting this could not be AIDS group. This could also probably explain the difference with results obtained from a study by Merlini and colleagues (2011). It may also explain why there was no difference in LPS levels between HIV-positive and HIV-negative participants with EE.

In a prospective longitudinal study, plasma 16S rRNA copy number were measured at baseline and at six months in HIV infected individuals who were on treatment and were compared to chronic untreated HIV infected individuals. Results at baseline and at six months showed both groups had 16S rRNA in plasma but the chronic untreated HIV infected individuals had significantly higher 16S rRNA suggesting a higher occurrence of MT in the chronic untreated HIV group (Chevalier *et al*., 2013). On the contrary, a different study compared plasma 16S rRNA copy number of HIV-infected adults at different stages of the disease: long-term non-progressors, treatment-naive patients with $CD4^+$ cell counts of greater than 350 but less than 500 cells/ µL, patients with $CD4^+$ cell count of <100 cells/µL who after receiving effective treatment achieved a

$CD4^+$ cell mean count of 1350 cells/μL, patients with $CD4^+$ cell count of <100 cells/μL who after receiving >2 years of effective treatment did not achieve a $CD4^+$ cell count of >200 cells/μL and healthy uninfected individuals. The results showed no significant difference between HIV-negative group and each one of the 4 HIV-positive groups (Ferri *et al*., 2010). Some studies have reported the presence of larger amount of 16S rRNA in plasma of healthy individuals whose origin still is unknown (Nikkari *et al*., 2001). In a similar study, Moriyama and colleagues (2008) reported presence of 16S rRNA gene copy number in healthy individuals. The 16S rRNA genes that was reported belonged to none of the known indigenous gastrointestinal flora. The researchers speculated that healthy individuals have 'normal' population of bacteria DNA in blood which have been partly hypothesized to be due to constant aspiration of airborne soil bacteria that when inhaled and destroyed by phagocytic cells or other immune cells in the respiratory system could later be carried into the circulatory system where they release their DNA in the blood stream.

In a randomized control trial, participants with HSS were divided into a group treated with rifaximin plus standard care and non-rifaximin (standard care only). After treatment, there was significant reduction in the 16S rRNA copy number in the group that was treated compared to the non-rifaximin group suggesting MT was reduced since the drug acts locally in the intestine (Sinkala *et al*., 2018). Real-quantitative PCR amplification of 16S rRNA gene in blood has been recommended and demonstrated in a number of studies (Sekirov *et al*., 2010; Kramski *et al*., 2011). It has been shown to be good enough as long as the primers used are designed to include highly conserved regions among different bacterial taxa. There have been different assays for detection of 16S rRNA that have been designed and available in literature, but the biggest challenge reported with the assay are bacterial contamination in the PCR reagents (Corless *et al*., 2000; Salter *et al*.,

2014). This has been known to result in a number of false positives which can prove difficult when it comes to clinical relevance of the assay (Novati *et al*., 2015). In this study, to address the issue of reagent and extraction kit contamination, all PCR master mixes were treated with 0.1 U of DNase I (Heininger *et al*., 2003). In studies that have reported higher 16S rRNA copies in HIV-positive individuals (Jiang *et al*., 2009, Cassol *et al*.,2010), PCR reagents and master mixes were not treated with enzymes that degrade DNA possibly leading to contamination and false positive.

5.4 Microbial Translocation and Toll-like receptor Ligands

Direct biomarkers of MT were significantly higher in both EE and HSS participants compared to healthy controls except for LPS which was found to be similar in HSS participants and healthy controls. It is difficult to measure MT and there is currently no single, non-invasive, validated biomarker available for this purpose. Most previous studies have used LPS and 16S rRNA genes as direct biomarker of MT (Marchetti *et al*., 2013; Bellot *et al*., 2013). This study also used TLRLs activity as a direct biomarker of MT. TLRLs have been used as a biomarker in many experimental conditions in LPS-stimulated RAW-Blue cells. In the assay secreted embryonic phosphatase (TLRLs) activity is measured in cell culture supernatant which indicates the presence of PAMPs that have caused stimulation.

TLRL activity was significantly higher in either the EE or HSS participants compared to healthy controls suggesting that there were more PAMPs in plasma of the EE or HSS participants due to MT and lead to the stimulation of RAW-Blue cells. There also was higher TLRLs in EE compared to HSS participants although the value in both groups were lower than the levels reported by other investigators who used LPS to stimulate RAW-Blue cells (Soromou *et al*., 2013; Park, 2014; Yoon *et al*., 2015). Other studies have also demonstrated that TLRL activity measurement in cell culture

supernatant after stimulation of RAW-Blue cells indicate the presence of PAMPs (Chang *et al*., 2013; Cheng *et al*., 2015; Leus *et al*., 2016). The discrepancy in results between this study and others studies could be due to passage number of cells used. In this study, cells between 4 and 7 passages were used while others (Soromou *et al*., 2013; Yoon *et al*., 2015) used cells between 1 and 3 passages. Cells undergo genotypic changes resulting in reduced responsiveness over time in culture conditions leading to reduced production of TLRL activity and subsequent cytokines. This could probably explain the differences between results from this study and those from others. However, RAW-Blue cells of less than 20 passages are recommended for these experiments (InvivoGen). The finding of high TLRL activity in participants with EE appears to support the hypothesis that people living in conditions of poor sanitation and hygiene are constantly exposed to fecal-oral microbial contamination leading to the establishment of T-cell mediated chronic inflammation in the intestine (Prendergast and Kelly, 2012). In this study, in participants with EE there was positive and significant correlation between TLRLs activity and biomarkers of host response to MT such as LBP, sCD14 and CD163. In participants with HSS TLRLs activity was correlated with LBP and sCD14 but in the healthy controls whereas TLRLs activity only correlated with LBP. Similar correlation results have been reported although not with TLRLs but LPS with sCD14 or LBP in HIV patients before treatment but disappeared after treatment suggesting that maybe direct biomarkers of MT were reduced after treatment (Abad-Fernández *et al*., 2013).

Studies in chronic HIV infection have suggested that LPS and bacterial DNA are not the only Toll-like receptor ligands that can lead to immune activation (Brenchley *et al*., 2006; Jiang *et al.,* 2009). TLRLs have been quantified in LPS-stimulated RAW-Blue cells in diverse contexts together with subsequence production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-

2 measured from cell culture supernatant (Zhou *et al*., 2011, Lewis *et al*., 2014). The major advantage of RAW-Blue cells is that it gives total PAMPs readout activity that is responsible for cell stimulation that are not currently measured by common assays (InvivoGen).

5.5 Microbial translocation and host immune response to microbial translocation

5.5.1 Microbial translocation and CD163

Circulating plasma sCD163 has been reported as a biomarker of Kupffer cell activation indicating host response to MT (Holland *et al*., 2011). In the current study, sCD163 was higher in participants with EE and HSS compared to healthy controls suggesting that there was more response to MT in both E and HSS participants than in the healthy controls probably due to the presence of direct biomarkers of MT. In patients with liver disease plasma sCD163 has been reported to be higher compared to healthy controls and was found to be a predictor of disease progression and mortality (Holger *et al*., 2007). Elevated sCD163 is an indication of host response to microbial translocation and it has been reported in other disorders such as inflammatory bowel diseases such as Crohn's disease (Pasternak *et al*., 2010) and ulcerative colitis (Pastor Rojo *et al*., 2007) and correlate with disease severity. In this study, sCD163 only correlated with TLRL activity in participants with EE. There were no significant correlation between sCD163 and any direct biomarker of MT in participants with HSS and healthy controls respectively. These results could suggest that the levels of direct biomarkers were not high enough to significantly elevate plasma CD163. In animal models such as non-human primates with chemically induced colitis (Hao *et al*., 2015), and in a mouse model where features of EE were triggered by diet and specific microbial exposure (Brown *et al*., 2015), reported elevated plasma sCD163 levels and attributed to MT due to compromised

intestinal barrier. Other studies have also used sCD163 as surrogate biomarker of MT in patients with cirrhotic portal hypertension (Holland-Fischer *et al*., 2011) while others have suggested it as a biomarker of tissue homeostasis and repair involved in immune modulation (Ellwardt and Zipp, 2014). In all these conditions there is compromised intestinal barrier which leads to MT such as in EE and HSS conditions. Elevated sCD163 has been reported in EE (Kelly *et al*., 2016) and significantly higher in HSS disease compared to healthy controls (Sinkala *et al*., 2016). In HIV infected individuals, whether on antiretroviral treatment or not, sCD163 levels have been reported to be significantly higher than those in HIV-negative individuals (Burdo *et al*., 2011). Although the precise function of sCD163 has not been clearly elucidated, it has been shown that during inflammatory conditions, macrophages are activated, resulting in the elevation of sCD163. Additional evidence has emerged pointing to sCD163 as a valuable biomarker of host response to MT as well as with a factor associated with progression of disease, morbidity and mortality in many inflammatory diseases (Mollar, 2012).

In this study, since sCD163 only correlated with TLRLs activity in participants with EE and not in the other groups. The levels of sCD163 in individuals with EE group and HSS did not differ but TLRLs were higher in EE than in HSS participants. This could probably explain why there was significant correlation between sCD163 and TLRLs only in the participants with EE. The difference between the results of this study and those from other studies could be due to differences in the study populations, assay techniques and study designs. Recently, another study reported that sCD163 in HIV-infected individuals is a predictor of mortality suggesting that intervention in such individuals should be targeted at controlling macrophages/monocytes activation (Knudsen *et al*., 2016). In other conditions such as alcohol-free liver disease it has been shown that sCD163 is associated with hepatic inflammation and fibrosis (Kazankov *et al*., 2014) and fibrosis has also

been suggested as one of the features of HSS (Mutengo *et al*., 2014, Sinkala *et al*., 2016). Findings from this study may suggest that targeting biomarkers of host immune response to MT could ameliorate individuals with EE or HSS.

5.5.2 Microbial Translocation and Lipopolysaccharide-binding protein

LBP is an acute phase protein that is mainly produced by hepatocytes in the liver. It is produced in response to LPS and therefore, it is used as a biomarker of host response to MT (Stehle *et al*., 2012). LBP binds LPS forming an LBP-LPS complex which is transported to membrane CD14 on macrophages or other cells of the innate immune system through TLR4 (Kim *et al*., 2016). This results in the production of pro-inflammatory cytokines (Schumann *et al*., 1996). This study found elevated LBP in both EE and HSS participants compared to healthy controls suggesting the MT in response to LPS (Ameno *et al*., 2000). Similarly, inflammatory bowel diseases such as Crohn's disease (Pastor Rojo *et al*., 2007) and ulcerative colitis (Pasternak *et al*., 2011) are characterized by chronic inflammation and compromised intestinal barrier function which leads to MT like in EE (Korpe and Petri, 2012) resulting in elevated LBP in response to LPS (Pastor Rojo *et al*., 2007). In this study, in individuals with EE, there was positive and significant correlation between LBP and LPS in keeping with what has been reported in IBDs (Lakato *et al*., 2011). Furthermore, another study demonstrated the use of LBP as a biomarker of host response to MT in patients with IBDs and found significantly higher levels these patients compared to healthy controls suggesting further evidence of MT (Funderburg *et al*., 2013); in agreement with the hypothesis that MT is a common feature in conditions with compromised intestinal barrier such as EE (Korpe and Petri, 2012) and in HSS patients (Sinkala *et al.,* 2016). A study in cirrhotic patients, found elevated LBP as well as considerable haemodynamic and immune derangements which after treatment with an

antibiotic, led to a reduction in LBP and correlated with clinical improvement. This was hypothesized to positively contribute to the reduction in MT (Albillos *et al*., 2003).

In this study, LBP positively and significantly correlated with all direct biomarker of MT (LPS, 16S rRNA and TLRL) in both EE and HSS participannts respectively. LPB was found to be directly correlated only with TLRLs in the healthy controls. These results could suggest that LBP is a good predictor of host response to MT in both EE and HSS. When participants with EE and HSS were combined a stronger correlation was obtained suggesting that if both conditions co-exist, LBP can become even better in predicting host immune response to MT. Similar results were reported in a study of patients with obstructive jaundice where MT has been reported to occur as well. Results from that study revealed the presence of elevated LBP which significantly decreased after draining and correlated with LPS (Kimmings *et al*., 2000). Overall, the results from this and other studies provide ample evidence that in conditions of compromised intestinal barrier function such as EE and HSS infection, MT is a major feature and some investigators have suggested that LBP is a better biomarker of MT than LPS because of its longer half-life. However, the major disadvantage is that it responds to LPS only and may not be useful as a biomarker for Gram-positive bacteria (Myc *et al*., 1997).

5.5.3 Microbial Translocation and soluble CD14

Soluble CD14 has been proposed as a surrogate biomarker for MT. Its target molecule is the LPS-LBP complex and its membrane form (mCD14), primarily expressed by macrophages. In the presence of LPS, its expression is increased in keeping with the concentration of LPS. In the detection of LPS, sCD14 together with TLR4 as a co-receptor binds LPS resulting in NF-κB activation (Landmann *et al*., 1996; Koutisonas *et al*., 2015). In this study, sCD14 levels were found to be higher in participants with EE compared to HSS or healthy controls. In participants with HSS it was also higher compared to healthy controls suggesting MT. Results from a recent study reported that patients with HSS had significantly elevated plasma sCD14 compared to healthy controls supporting its role as a biomarker of microbial translocation. In individuals with EE, sCD14 levels were elevated more than the reference range suggesting response to MT (Kelly *et al*., 2016). These results were in consistent with the findings from this study that in EE compromised intestinal barrier function lead to MT. In patients with liver disease, sCD14 was reported to correlate with 16S rRNA suggesting MT (Tuomisto *et al*., 2015). These results were similar to those found in this study. Further, in other studies in patients with hepatitis B virus (HBV) and hepatitis C virus (HCV) infection have suggested that sCD14 is not only a good biomarker of MT but also a biomarker of disease progression (Sandler *et al*., 2011). This was supported by similar findings in HIV patients with disease progression co-infected with HCV (French *et al*., 2013). Furthermore, a good correlation of sCD14 with other biomarkers such as LPS and LBP was reported in long-term suppressed HIV-1–infected individuals. In the current study, higher plasma sCD14 were observed in participants with EE or HSS participants compared to healthy controls further support the model that MT occurs in individuals with EE and HSS.

5.5.4 Microbial translocation and C-reactive protein

CRP is considered to be an acute phase reactant protein produced by the liver. Higher CRP levels are produced by macrophages in response to non-specific inflammation (Thompson *et al*., 1999) . In this study, the levels of CRP were similar among participants with EE, HSS and healthy controls. This may suggest that since CRP is an acute phase protein, it might not be a sensitivity biomarker of inflammation in both EE and HSS conditions which are both chronic conditions. This is in keeping with what others have reported in EE (Kelly *et al*., 2016) and in HSS patients (Sinkala *et al*., 2016). Further, significant correlations between CRP with any other biomarker could not be established. This requires further investigation especially that these conditions are common and may co-exist in tropical countries such as Zambia. On the contrary, in IBDs, CRP was reportedly elevated and significantly correlated with biomarker of MT as well as disease progression (Lakato *et al*., 2011).

In this study there was no difference in biomarkers of host response to MT between EE and HSS participants except for sCD14. This could suggest that significantly elevated LPS which was observed in participants with EE more than in those with HSS could activate Kupffer cells by interacting with the membrane CD14 and MD2/TLR4 resulting in the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-2. This could also explain the pattern of cytokines observed more in EE than HSS participants or healthy controls.

5.6 Microbial Translocation and Cytokines

RAW-Blue cells are mouse macrophages that produce inflammatory cytokines upon stimulation by various PAMPs (Soromou *et al*., 2013; Wang and Cao, 2014). They are widely used to establish inflammatory models *in vitro* (Krenn *et al*., 2009; Zhang *et al*., 2010). Inflammation is beneficial to the host as it leads to the elimination of infectious agents (Szliszka *et al*., 2011). However, if it is not controlled inflammatory mediators can lead to microcirculatory dysfunction, tissue damage, angiogenesis, and septic shock common in many chronic inflammatory diseases (Yoon *et al*., 2009). This study investigated the production of cytokines by plasma-stimulated RAW-Blue cells from cell culture supernatants and the findings are discussed in the following sub-sections.

5.6.1 Microbial Translocation and Tumor Necrosis Factor-alpha

TNF-α is a major pro-inflammatory cytokine produced by various immune cells including macrophages in response to stimuli (Laveti *et al*., 2013). This study found significantly higher TNF-α in cell culture supernatant in participants with EE compared to those with HSS or healthy controls and the levels were also higher in participants HSS than in healthy controls. These results suggest that in plasma of individuals with EE there were more PAMPs that caused stimulation of the RAW-Blue cells compared to the participants with HSS or healthy controls due to MT. Elevated levels of TNF-α have been shown to reduce expression of tight junction proteins such as zonula occludens and claudins leading to increased permeability and MT (Clayburg *et al*., 2009; Shen *et al*., 2011). Since in both participants with EE and HSS had elevated TNF-α, it is possible that MT might be aggravated by the increased TNF-α levels in these groups similar to what has been reported in IBDs (Zeissing *et al*., 2007). Conflicting results and interpretations have been reported. Some investigators have suggested that TNF-α alone might not have a profound effect on the tight junction and may require a much greater cellular disruption by other means (Pasternak

et al., 2010). Results from other studies have reported that TNF-α alone is enough to cause enteropathy (Garside *et al*., 1993).

This study also investigated the relationship between TNF-α and direct biomarkers of MT (LPS, 16S rRNA and TLRL). There were positive and significant correlations between TNF-α and LPS, 16S rRNA as well as TLRL in both EE and HSS participants but not in the healthy control group suggesting that all direct biomarkers significantly predicted TNF-α except LPS in participants with after adjustment for baseline characteristics in the multivariate multiple regression model. Studies have reported that increased TNF-α levels in serum and intestinal tissues of patients with Crohn's disease and other inflammatory diseases correlate with severity of the disease (Ito *et al*., 2003; Serhan *et al*., 2008). In keeping with results from other studies, this study suggests that the higher the biomarkers of MT and the TNF-α, the stronger the correlations suggesting that a rise in the direct biomarkers was predicting the levels of TNF-α.

5.6.2 Microbial Translocation and Interleukin-6

IL-6 is a prototypic pro-inflammatory cytokine produced by various immune cells in response to inflammation and infection (Laveti *et al*., 2013; Wang and Cao, 2014). This study found higher levels of IL-6 in individuals with EE compared to HSS participants or healthy controls. This suggests that there were more PAMPs in plasma of participants with EE compared to HSS or healthy controls leading to production of IL-6. A study in a mouse model found diminished intestinal permeability after treatment with a specific monoclonal antibody to IL-6. This resulted also in reduction in the expression of claudin-2 indicating that inhibition of IL-6 promotes

functional intestinal barrier integrity (Xiao *et al*., 2016). Another study showed that in IBDs, IL-6 enhances intestinal permeability through mechanisms believed involving mitogen-activated protein kinase (MEK), extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways in intestinal epithelial cells. This was hypothesized to occur by increasing the expression of channel-forming claudin-2 (Suzuki *et al*., 2011). In this study, the levels of IL-6 were found to be positively and significantly correlated with all direct biomarkers of MT (LPS, 16S rRNA and TLRLs) in both participants with EE and HSS but not in healthy controls respectively. This suggests that LPS, 16S rRNA and TLRLs could have led to production of IL-6 consistent with results from other studies that have reported that increased IL-6 correlates with disease severity in inflammatory diseases such as Crohn's disease (Funderburg *et al*., 2013) and in cancer development (Cook *et al*., 2013).

5.6.3 Microbial Translocation and Interleukin-10

IL-10 is known to be a pleiotropic cytokine with predominantly suppressive effects on pro-inflammatory cytokines mainly produced by macrophages and monocytes (de Waal *et al*., 2000). In the current study, IL-10 levels were higher in participants with EE compared to HSS or healthy controls. In mouse models of IBDs, studies have reported that IL-10 has anti-inflammatory properties and showed improved intestinal architecture after treated with IL-10 (Wojdasiewicz *et al*., 2014; Kessler *et al*., 2017). This study found higher levels of IL-10 as well as pro-inflammatory cytokines (TNF-α and IL-6) in both participants with EE and HSS suggesting that IL-10 was unable to reduce inflammatory response. It could be that since there was increased production of TNF-α and IL-6 in EE and HSS participants, pro-inflammatory cytokines overweighed the suppressive effect of IL-10. Since IBDs are characterized by chronic intestinal inflammation

similar to EE (Korpe and Petri, 2012) and in HSS patients' chronic inflammation is also a common feature (Sinkala *et al*., 2016); and this study found elevated IL-10 in both EE and HSS together with pro-inflammatory cytokines compared to healthy controls, this could suggest that IL-10 did not reduce the effect of pro-inflammatory cytokines. This could mean that the process of inflammation could continue despite elevated IL-10 levels in both the EE and HSS participants. In agreement with this study, a double-blind and placebo controlled study which was set out to explore the efficacy and safety of IL-10 treatment in patients with crohn's disease reported minor and insignificant clinical improvement (Schreiber *et al*., 2000) suggesting treatment with IL-10 did not alleviate the disease.

Further, another study reported that in patients with autoimmune diseases, lupus and encephalomyelitis, IL-10 is involved in the abnormal angiogenesis and pathobiological processes (Ishihara *et al*., 2002) suggesting minimal anti-inflammatory function. In an animal model, a study was carried out in macaques to describe the pathogenesis of HIV-mediated enteropathy and found that enhanced production of IL-10 was consistent with the disruption of intestinal barrier indicated by loss of ZO-1 tight junction protein and was linked to increased production of pro-inflammatory cytokines such as TNF-α and INF-γ. The study concluded that probably IL-10 was unable to down regulate inflammatory response in agreement with findings of this study. Although IL-10 is traditionally believed to be a potent repressor of pro-inflammatory cytokines, it seems the clear role of IL-10 remains to be elucidated going by the conflicting results reported from different studies. Some have reported that IL-10 suppresses production of pro-inflammatory cytokines (Pradervand *et al*., 2006) while others have suggested that it has capacity to stimulate cells that are involved in the establishment of inflammation such as T cells, natural killer cells and B-cells (Mocellin *et al*., 2004). Some key findings on IL-10 trial in humans have been inconsistent as well.

IL-10 treatment of patients with Crohn's disease showed no clear evidence of clinical improvement (Colombel *et al*., 2001), similar to what others reported in patients with active Crohn's disease (Schreiber *et al*., 2000). Results from other studies have however, reported improved clinical response and intestinal endoscopic appearance (Fedorak *et al*., 2000). The inconsistencies in the role of IL-10 could be due to differences in the populations studied, research designs, environmental conditions, variations in ages of participants and severity of the disease. In this study, IL-10 positively and significantly correlated with all direct biomarkers of MT (LPS, 16S rRNA and TLRLs) in both EE and HSS participants but not in the healthy controls suggesting that direct biomarkers predicted production of IL-10 or the higher the values the more likely the correlation. In both animal and *in vitro* models some studies have shown that administration of IL-10 correlates with amelioration of inflammation in chronic enterocolitis, which is similar to human Crohn's disease (Aithal *et al*., 2001; Marlow *et al*., 2013). This suggests that higher IL-10 reduces inflammation. On the contrary, in this study there was higher levels of IL-10 as well as pro-inflammatory cytokines in both EE and HSS participantnts indicating that inflammatory process in these groups was not down regulated.

5.6.4 Microbial Translocation and Interleukin-2

IL-2 is a pro-inflammatory cytokine that has been reported to be elevated in diseases involving inflammatory processes (Akbar 1996; Sheller *et al*., 2009). This study found higher levels of IL-2 in both EE and HSS participants compared to healthy controls. Another study reported increased IL-2 levels in LPS-stimulated RAW-Blue cells in cell culture supernatant (Park, 2014). In this study, higher levels of IL-2 in both EE and HSS participants could suggest that there were more PAMPs in plasma that led to IL-2 stimulation due to MT. However, in the multivariate multiple

regression models while controlling for baseline characteristics, all direct biomarkers did not predict IL-2 possibly the study was underpowered to find the difference if it exists.

5.6.5 Microbial Translocation and Interleukin-4

IL-4 is regarded as an anti-inflammatory cytokine and therefore, is involved in resolution of inflammation (East and Isacke, 2002). In this study, IL-4 levels were significantly higher in both EE and HSS participants compared to healthy controls. In mouse model of colitis, a study reported reduced levels of IL-4 and increased levels of IFN-γ, a pro-inflammatory cytokine in mice with colitis compared to healthy controls (Liu *et al*., 2008) suggesting that IL-4 did not reduce the pro-inflammatory cytokine. On the contrary, a different study reported increased expression of IL-4 mRNA levels in chronic inflammation (Desreumaux *et al*., 1997). In another colitis model, Choi *et al*., (2010) reported increased expression of IL-4 mRNA in colitis induced mice compared to healthy controls. In humans with both Crohn's disease and ulcerative colitis, elevated levels of IL-4 have been reported in mucosal immune cell cultures (Fiocchi *et al*., 1998). Since this study found higher levels of IL-4 in both EE and HSS participants compared to the healthy controls, suggesting that levels of IL-4 were not sufficient to reduce the levels of pro-inflammatory cytokines. Since other studies have shown that IL-4 seems not to be consistent, it could play a role similar to pro-inflammatory cytokines in both EE and HSS infection. These inconstancies on the role of IL-4 in different conditions warrant further investigation in order to understand its immune mechanism. One of the unexpected results was lack of correction between IL-4 and direct biomarkers of MT. This may be due to its role is not yet elucidated properly, confounding variables or it could also be that the levels were not high enough for corrections to be significant.

5.6.6 Microbial Translocation and Interleukin-17A

IL-17A is a pro-inflammatory cytokine mainly produced by various immune cell types. Examples of IL-17A producer include macrophages, dendritic cells and natural killer T cells. It has been shown to increase recruitment of neutrophils and monocytes to the site of inflammation as well as stimulate chemokine production (Mangan *et al*., 2006). In this study plasma-stimulated RAW-Blue cells did not produce IL-17A in all the groups studied as well as IFN-γ. Some studies have reported considerable amount of IL-17A produced by activated macrophages after stimulation with LPS (Park, 2014; Soromou *et al*., 2012). In inflammatory conditions such as Crohn's disease some studies have reported increased production of intestinal mucosal IL-17A (Sakuraba *et al*., 2009; Kleinschek *et al*., 2009) although it is still not clear whether IL-17A contribute to Crohn's disease. In an animal model IL-17A has been shown to induce colitis (Hao *et al*., 2015). However, a different mouse model demonstrated that treatment of IL-17A knockout mice with dextrane sulfate sodium (DSS) failed to induce acute colitis (Ito *et al*., 2009) but induced colitis in animals with IL-17A suggesting that IL-17A has a pro-inflammatory role (Zhang *et al*., 2008). This study used LPS as a control at maximum concentration of 1000 ng/ml. There was no IL-17A production even at lower concentrations in the controls. This finding was in agreement with what has been reported elsewhere (Lee *et al*., 2012) after LPS-stimulated RAW-Blue cells. On the contrary, other studies have reported production of IL-17A in LPS-stimulated RAW-Blue macrophages (Yoon *et al*., 2009; Szliszka *et al*., 2013).

In a study by Yoon *et al*. (2009) the concentration of LPS (1000ng/ml) used stimulate RAW-Blue cells was similar to what this study used but there were differences. First, this study used cells between 4 to 7 passages but Yoon and colleagues did not report cell passage number used which is important because cells undergo genotypic changes resulting in reduced responsiveness over

time which is normal in cell culture conditions (InvivoGen). Second, Yoon *et al.* (2009) used a higher concentration of cells (2 x 10^5 cells per well) while this study used 1 x 10^5. The inconsistencies that have been reported necessitate further studies to establish whether IL-17A is produced by stimulated RAW-Blue macrophages.

To date, there is no single biomarker that can be used as gold-standard to measure and confirm MT in EE or HSS and due to the complex nature of the conditions, it is very improbable that a single biomarker would even be identified or developed to describe EE. A wide range of biomarkers have to be considered in order to provide insights into EE and its consequences. Since this was an observational study inference about causality could not be made. However, the findings are in line with the hypothesis that in individuals with EE and HSS MT take place which lead to chronic inflammation followed by heightened and persistent immune activation.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, this study found higher levels of plasma LPS, 16S rRNA, and TLRL in individuals with EE compared with HSS patients or healthy control. Plasma sCD14 and sCD163 were also elevated in EE compared with HSS patients or controls. In both EE and HSS patients, the

correlation of direct biomarkers with immune activation measured by TNF-α, IL-16, and IL-10 was significant. These data are in line with a model that biomarkers of microbial origin in the gastrointestinal tract move across a compromised intestinal barrier in EE and HSS patients, inducing systemic immune activation.

6.2 Recommendations

The study recommends measurement of MT using three direct biomarkers LPS, 16S rRNA and TLRLs is cheaper, practical and non-invasive compared to culturing MLNs which is invasive and not practical to sample all MLNs in EE and HSS infection and probably other condition where microbial translocation occur. Although endoscopy is a gold-standard to confirm EE, it is invasive, expensive and need specialised personnel so these biomarkers can be an alternative to measure MT. Clearly, more research is needed for further evidence to support the current findings, especially with the use of RAW-Blue cells to detect total PAMPs. This study found elevated IL-10, anti-inflammatory cytokine together pro-inflammatory cytokines contrary to most literature. It is, therefore, recommended that further studies be conducted to provide more insights in the mechanism of IL-10 especially in EE and HSS infection. Both the EE and HSS participants had consistently higher cytokines compared to healthy controls. The management of individuals with EE and HSS infected individuals should incorporate the analysis of some or all of the cytokines used in this study. The main strength of the current study was that it used three different approaches to measure MT which are practical and inexpensive and all biomarkers showed consistent results. The consistence was followed by biomarker of host response to MT and immune activation suggesting evidence and reliable data that MT occurs in EE and HSS participants.

6.3 Recommendations for Future Research

Further research is needed to confirm which microbes are responsible for translocation and what are their effects on the immune system. Further, there is need to establish how does cytokines activity *in-vivo* lead to enteropathic changes? Is there a vicious cycle? Also there is need to establish which TLRs predominantly activated in EE and HSS?

Furthermore, research is needed to confirm links between LPS, 16S rRNA and TLRLs in the EE and HSS infected individuals and immune activation. This is because these direct biomarkers independently predicated TNF-α, IL-6 and IL-10 these groups except LPS in the HSS participants. The use of RAW-Blue cells macrophages need to be strengthened in our setting as it has shown potential to detect PAMPs in plasma.

6.4 Contribution of Knowledge to Science

As far as literature was searched this is the first study to measure all possible direct biomarkers of MT in both EE and HSS infected individuals and compared the two groups. These biomarkers were predictors of immune activation.

6.5 Application of Results in Public Health

The reported elevated direct biomarkers of MT, biomarkers of host immune response to MT and cytokines in both EE and HSS participants may necessitate use of these biomarkers to monitor individuals with these conditions. These findings may help design and deliver much-needed robust evaluation of biomarkers for better management and permit assessment of interventions in individuals with EE and HSS participants. Since intestinal changes in both EE and HSS participants can only be known by endoscopy which is invasive, expensive and not readily available in most health care centres, these biomarkers which are cheaper and non-invasive could

be possibly be used to monitor and predict the outcome especially in resource-poor settings such as Zambia.

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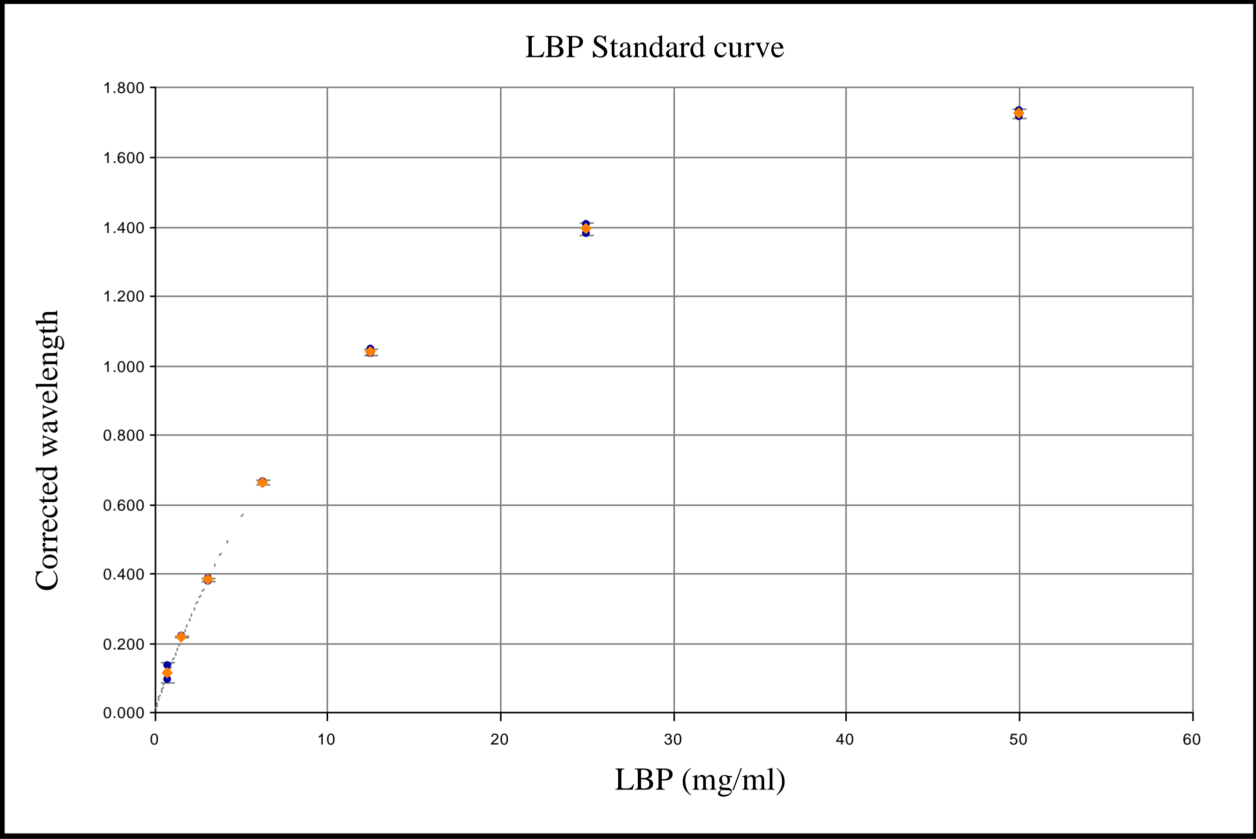
APPENDIXES

APPENDICE I

Lipopolysaccharide-binding protein standard dilution curve

Curve Name	Curve Formula	Parameter	Value	Std. Error	95% CI min	95% CI max
Curve	Y = (A-D) /(1+(X/C)^B) + D	A	0.00915	0.0246	-0.0692	0.0875
		B	1.02	0.0588	0.833	1.21
		C	14.4	0.964	11.3	17.4
		D	2.2	0.0784	1.95	2.45

Well ID	Well	Conc. from kit insert	Corrected wavelength	Conc. obtained	Count	Mean	Std Dev	CV (%)
STD1	G1	50	1.716	49.313	2	50.577	1.788	3.54
	H1	50	1.735	51.842				
STD2	G2	25	1.382	23.837	2	24.424	0.83	3.4
	H2	25	1.408	25.011				
STD3	G3	12.5	1.035	12.649	2	12.798	0.211	1.65
	H3	12.5	1.048	12.947				
STD4	G4	6.25	0.661	6.172	2	6.219	0.066	1.06
	H4	6.25	0.667	6.265				
STD5	G5	3.13	0.382	3.03	2	3.064	0.048	1.56
	H5	3.13	0.388	3.098				
STD6	G6	1.56	0.22	1.6	2	1.592	0.012	0.73
	H6	1.56	0.219	1.584				
STD7	G7	0.781	0.096	0.634	2	0.784	0.213	27.1
	H7	0.781	0.137	0.935				

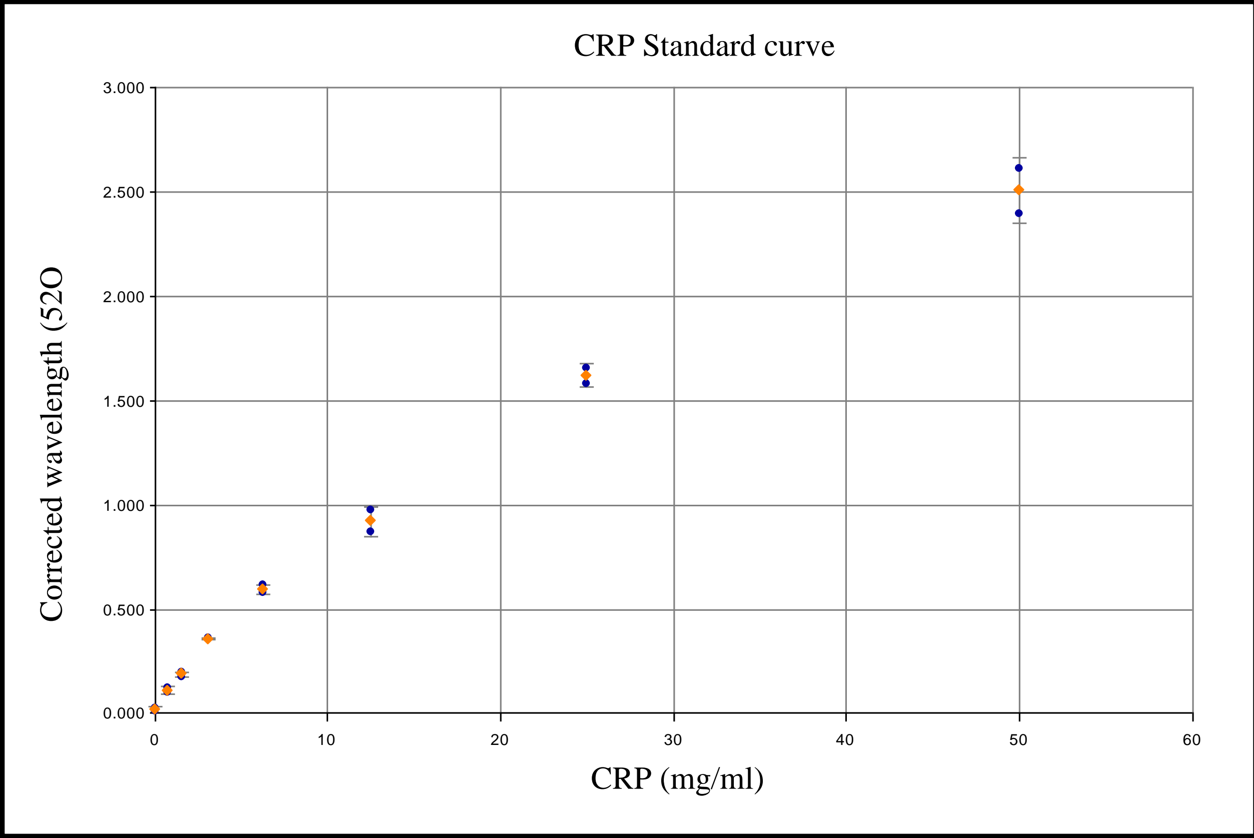


Standard curve for LBP obtained from using the standards provided in the kit. All the values obtained were within the manufacturer's acceptable values. After obtaining the expected values then the samples were run in duplicates.

Appendix II: C-reactive protein standard dilution curve

Curve Name	Curve Formula	Parameter	Value	Std. Error	95% CI min	95% CI max
Curve	Y = (A-D) /(1+(X/C)^B) + D	A	0.0171	0.0287	0.0626	0.0969
		B	0.84	0.0837	0.607	1.07
		C	168	140	-219	555
		D	9.42	4.22	-2.31	21.1

Well ID	Well	Conc. from the kit	Corrected wavelength	Conc. obtained	Count	Mean	Std Dev	CV (%)
STD 1	A1	50	2.39	48.32	2	50.32	1.54	3.43
	A2	50	2.62	52.50				
STD 2	B1	25	1.58	24.71	2	25.59	1.25	4.87
	B2	25	1.66	26.47				
STD 3	C1	12.5	0.97	12.55	2	12.68	1.23	4.5
	C2	12.5	0.87	11.82				
STD 4	D1	6.25	0.58	6.34	2	6.58	0.34	5.1
	D2	6.25	0.62	6.81				
STD 5	E1	3.12	0.36	3.40	2	3.37	0.04	1.28
	E2	3.12	0.36	3.34				
STD 6	F1	1.56	0.18	1.35	2	1.45	0.14	9.56
	F2	1.56	0.19	1.54				
STD 7	G1	0.78	0.13	0.84	2	0.72	0.18	25
	G2	0.78	0.09	0.58				
STD 8	H1	0	0.01	0.00	2	0.05	1.04	3.24
	H2	0	0.03	0.05	2	0.08	1.09	

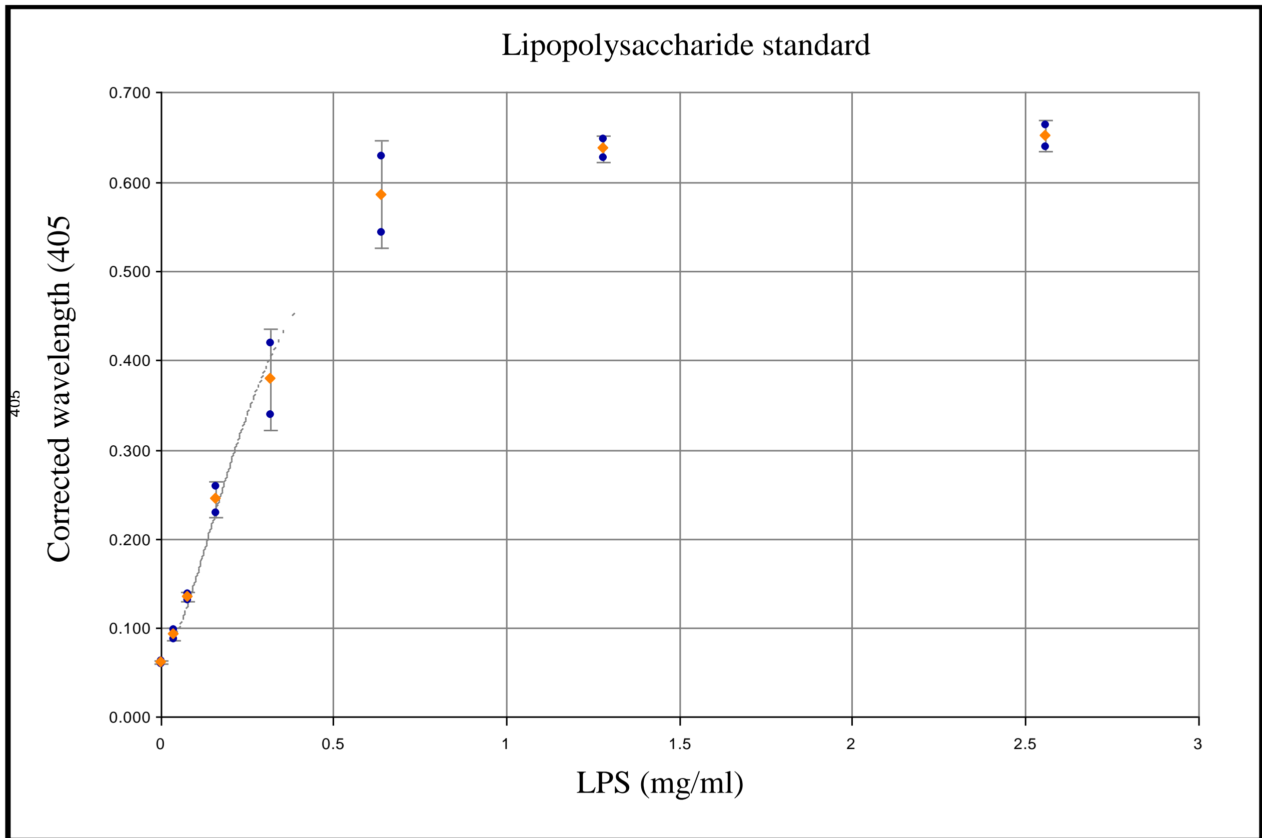


Standard curve for CRP obtained from using the standards provided in the kit. All the values obtained were within the manufacturer's acceptable values. After obtaining the expected values then the samples were run in duplicates.

Appendix III: Lipopolysaccharide standard dilution curve

Curve Name	Curve Formula	Parameter	Value	Std. Error	95% CI min	95% CI max
Curve	Y = (A-D) /(1+(X/C)^B) + D	A	0.0713	0.0176	0.0224	0.12
		B	1.77	0.245	1.09	2.45
		C	0.283	0.0238	0.217	0.349
		D	0.675	0.0218	0.615	0.736

Well ID	Well	Conc. from the kit	Corrected Wavelength	Conc. obtained	Count	Mean	Std Dev	CV (%)
STD1	A1	2.56	0.67	2.68	2	2.57	0.01	2.43
	A2	2.56	0.64	2.46				
STD2	B1	1.28	0.65	1.58	2	1.35	0.32	13.9
	B2	1.28	0.63	1.12				
STD3	C1	0.64	0.54	0.58	2	0.87	0.40	12.4
	C2	0.64	0.63	0.61				
STD4	D1	0.32	0.34	0.25	2	0.29	0.06	11.1
	D2	0.32	0.42	0.34				
STD5	E1	0.16	0.26	0.18	2	0.17	0.02	9.38
	E2	0.16	0.23	0.16				
STD6	F1	0.08	0.13	0.08	2	0.09	0.01	5.61
	F2	0.08	0.14	0.09				
STD7	G1	0.04	0.09	0.04	2	0.05	0.01	13.3
	G2	0.04	0.09	0.05				
STD8	H1	0	0.06	0.00	2	0	0	0
	H2	0	0.06	0.00				



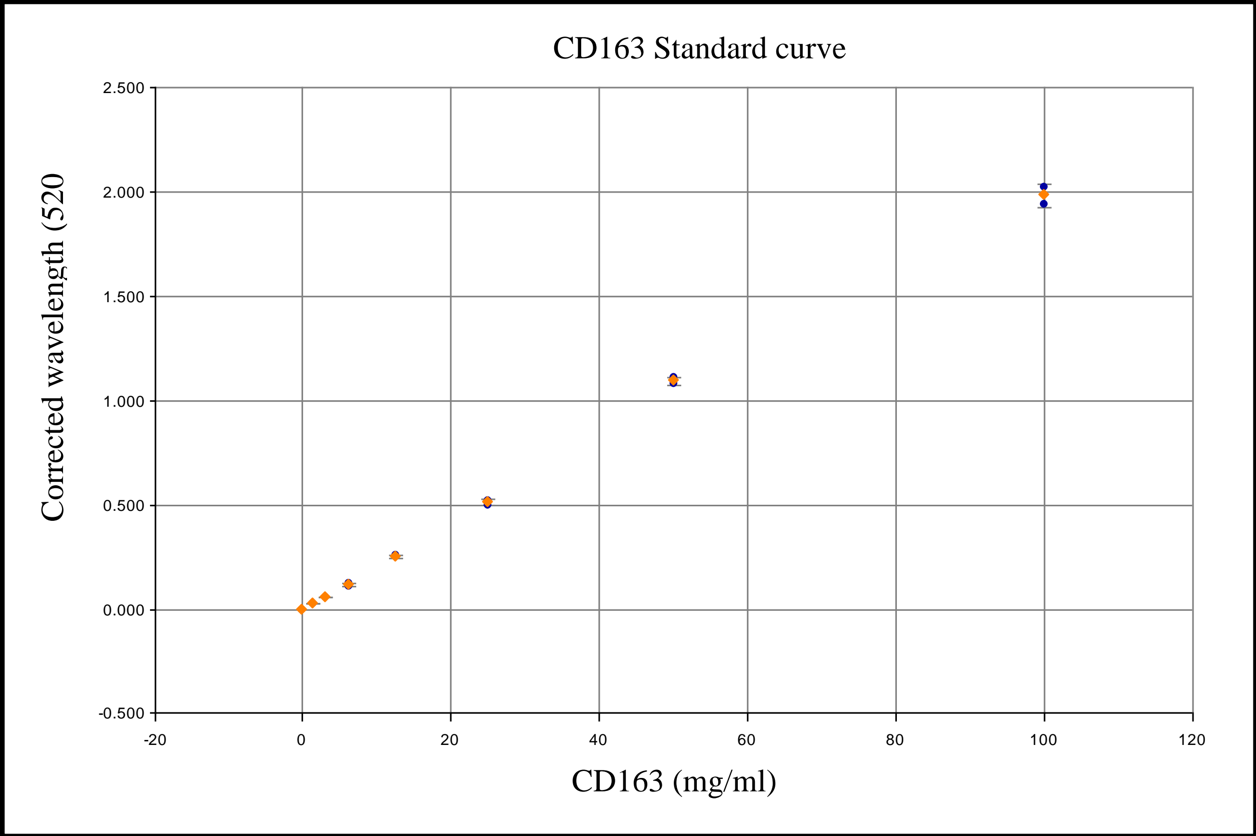
Standard curve for LPS obtained from using the standards provided in the kit. All the values obtained were within the manufacturer's acceptable values. After obtaining the expected values then the samples were run in duplicates.

Appendix IV: Soluble CD163 standard dilution curve

Curve Name	Curve Formula	A	B	R^2
Curve	Y=A*X+B	0.0202	0.00561	0.998

Curve Name	Curve Formula	Parameter	Value	Std. Error	95% CI min	95% CI max
Curve	Y=A*X+B	A	0.0202	0.000411	0.0192	0.0212
		B	0.00561	0.0168	0.0355	0.0467

Well ID	Well	Conc. from the kit	corrected wavelength	Conc. obtained	Count	Mean	Std Dev	CV (%)
STD1	G1	100	1.94	95.99	2	98.07	2.94	3
	H1	100	2.03	100.15				
STD2	G2	50	1.08	53.31	2	54	0.98	1.82
	H2	50	1.12	54.69				
STD3	G3	25	0.50	24.61	2	25.10	0.70	2.79
	H3	25	0.522	25.59				
STD4	G4	12.5	0.25	12.12	2	12.36	0.35	2.84
	H4	12.5	0.26	12.61				
STD5	G5	6.25	0.11	5.13	2	5.45	0.46	8.37
	H5	6.25	0.12	5.77				
STD6	G6	3.13	0.06	2.49	2	2.55	0.07	2.75
	H6	3.13	0.06	2.59				
STD7	G7	1.5	0.03	0.96	2	1.09	0.18	16.2
	H7	1.5	0.03	1.21				
STD8	G8	0	0.01	0.00	0	0	0	0
	H8	0	0.01	0.00				

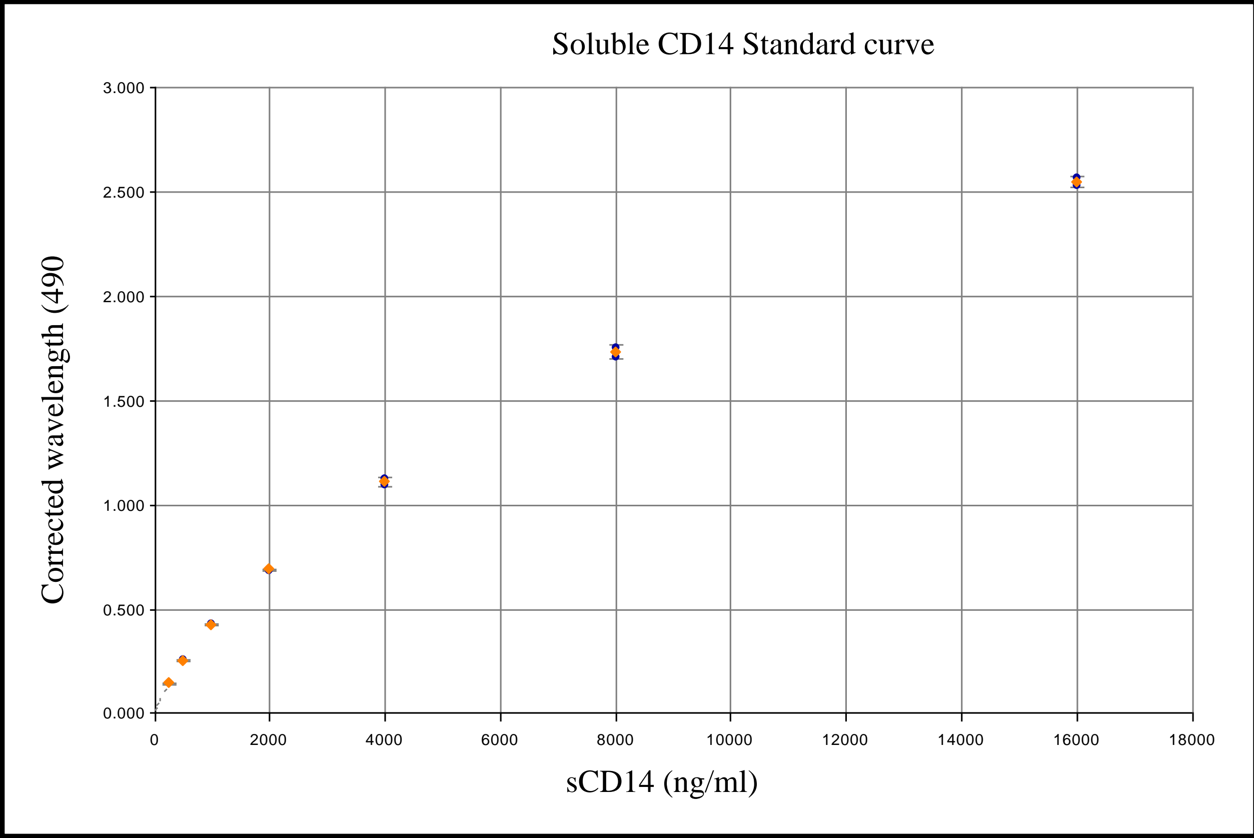


Standard curve for CD163 obtained from using the standards provided in the kit. All the values obtained were within the manufacturer's acceptable values. After obtaing the expected values then the samples were run in duplicates.

Appendix V: Soluble CD14 standard dilution curve

Curve Name	Curve Formula	Parameter	Value	Std. Error	95% CI min	95% CI max
Curve	Y = (A-D) /(1+(X/C)^B) + D	A	0.000897	0.0135	-0.0419	0.0437
		B	0.781	0.024	0.705	0.858
		C	3.72E+04	6.62E+03	1.61E+04	5.83E+04
		D	7.48	0.616	5.52	9.44

Well ID	Well	Conc. from the kit	corrected Wavelength	Conc. obtained	Count	Mean	Std Dev	CV (%)
STD1	G1	16000	2.53	15770.24	2	15989.5	310.09	1.94
	H1	16000	2.57	16208.76				
STD2	G2	8000	1.72	7857.66	2	8031.32	245.59	3.06
	H2	8000	1.76	8204.99				
STD3	G3	4000	1.09	3897.34	2	3978.15	114.27	2.87
	H3	4000	1.13	4058.95				
STD4	G4	2000	0.67	1973.99	2	1988.24	20.16	1.01
	H4	2000	0.69	2002.49				
STD5	G5	1000	0.43	1024.18	2	1016.03	11.52	1.13
	H5	1000	0.42	1007.89				
STD6	G6	500	0.25	503.96	2	507.96	5.66	1.11
	H6	500	0.26	511.96				
STD7	G7	250	0.15	244.74	2	241.43	4.67	1.93
	H7	250	0.14	238.13				



Standard curve for sCD14 obtained from using the standards provided in the kit. All the values obtained were within the manufacturer,s acceptable values. After obtaing the expected values then the samples were run in duplicates.

APPENDIX VI: Consent form

Direct biomarkers of microbial translocation as predictors of immune activation in adult Zambians with Environmental Enteropathy and Hepatosplenic Schistosomiasis

Consent record form (Kept by Researcher)

I confirm that I have read and fully understood the information I have been presented with about the whole study. I agree to participate in the study. I confirm that I am joining the study out of my free will without being influenced will that I can withdraw at any time I feel like without affecting my rights of medical care available to me. I understand what will be required of me.

Name:………………………………………………..

Signed (or thumbprint)

Date:…………………………………………………

Signature (or thumbprint) of witness

Name:……………………………………………….

Date:………………………………………………..

I, the member of the study team, I confirm that I have explained the information fully and answered any questions

Signed for the study team

Name:………………………………………………

Date:……………………………………………….

APPENDIX VII: Information Sheet for Participants

Direct biomarkers of microbial translocation as predictors of immune activation in adult Zambians with environmental enteropathy and hepatosplenic schistosomiasis

Information sheet

We have invited you to participate in a study that is designed to measure total direct biomarkers of microbial translocation and whether they predict immune activation in individuals with environmental enteropathy and hepatosplenic schistosomiasis.

Some previous studies have shown that microbial translocation takes place in environmental enteropathy and hepatosplenic schistosomiasis patients but none of these studies have quantified the total biomarkers and determine whether they are predictors of immune activation in these individuals.

Therefore, in this study we want to find out whether direct biomarkers of microbial are predictors of immune activation and also whether they correlate with each other and immune activation. Results of this study will be used to deliver much-needed robust evaluation of biomarkers for better management and permit assessment of interventions in both environmental enteropathy and hepatosplenic schistosomiasis.

What are we asking you to do?

If you are agree to participate in this study, we will collect 10mls of blood which is equivalent to approximately 2 table spoons using a needle. The blood collection procedure is generally safe but with a little discomfort and a bit of pain at the time of pricking into the vein on your arm. However, this pain does not last for a long time it goes within few minutes. The whole procedure will not take more than 15 minutes of your time. You are required to do an HIV test but before that you

need to be counseled. Generally, counseling before and after testing will take approximately maximum time of about 30 minutes.

What are we going to do with your samples?

The blood samples that we will collect from you will be used for testing HIV, full blood count, lipopolysaccharide, 16S rRNA and Toll-like receptor ligands, soluble CD14, CD163, lipopolysaccharide-binding protein and C-reactive protein.

Are there possible benefits to me?

In an event that we find anything that need medical attention, it will be provided to you free of charge.

Are there any possible disadvantages to me?

The disadvantage may be the little pain you feel when getting blood with the needle from your arm and the time itself that will spend the whole procedure.

Confidentiality

The results of all the study will be discussed with you, and kept confidential. All information obtained in this study will be considered confidential and used only for research purposes. No one will be allowed to access any information related to you.Your details will be recorded on the sheet with be kept secure and your name will not be included only the study number.

Injury clause

In the event that you become injured during the course of the research study, immediately notify the principal investigator or the chairperson of the Research Biomedical Ethical committee of the University of Zambia, School of Medicine on telephone number 0211256067 or P.O BOX 50110, Ridgeway campus, Lusaka. If you believe that your injury directly resulted from the search procedures of this study, you can file a complaint with the principal investigator. For a description

of this process, contact the Chairperson of Research Biomedical Ethics Committee at University of Zambia, School of Medicine on telephone 0211256067.

Right to Refuse or Withdraw

Your participation in the study is entirely voluntary, and you are free to refuse to take part or withdraw at any time without affecting or jeopardizing your future medical care.

Contact details of the Principal investigator:

Mr Patrick Kaonga

Tropical Gastroenterology & Nutrition Group (TROPGAN), Department of Internal Medicine, University of Zambia School of Medicine, UTH, Nationalist Road P.O. BOX 50398, Lusaka, Zambia (Phone 0977349386)

Contact details of Research Ethics Committee:

The Chairperson

Research Biomedical Ethics Committee office

Department of Anatomy, Ridgeway Campus, Nationalist Road, Lusaka

(Phone 0211 256067) 228

APPENDIX VIII: Questionnaire

Direct biomarkers of microbial translocation as predictors of immune activation in adult Zambians with Environmental Enteropathy and Hepatosplenic Schistosomiasis

Questionnaire Study ID NO.____________

1. Date:

2. Age (years) ___________________

3. Sex

(a) Male

(b) Female

4. Religion

(a) None

(b) Christian

(c) Muslim

(d) Hindu

(e) Other____________________

5. Occupation

(a) Unemployed

(b) Employed

6. What is you highest education level completed?

(a) No education

(b) Primary

(c) Secondary

(d) Tertiary

7. What is your marital status?

(a) Married

(b) Widow/widower

(c) Divorced

(d) Single-never married

(e) Other____________________

7. General Health and well-being

a. Have you used any antibiotic for the past one month?

b. Have you had diarrhea for the past two weeks?

Yes /No

If yes, for how long _____________(Days)

8. Blood Sample

a. Specific time of collection ______:_____

b. Any problems during blood collection ____________

c. Specific time received in the laboratory ______:_____

d. What is the HIV test result? _________________

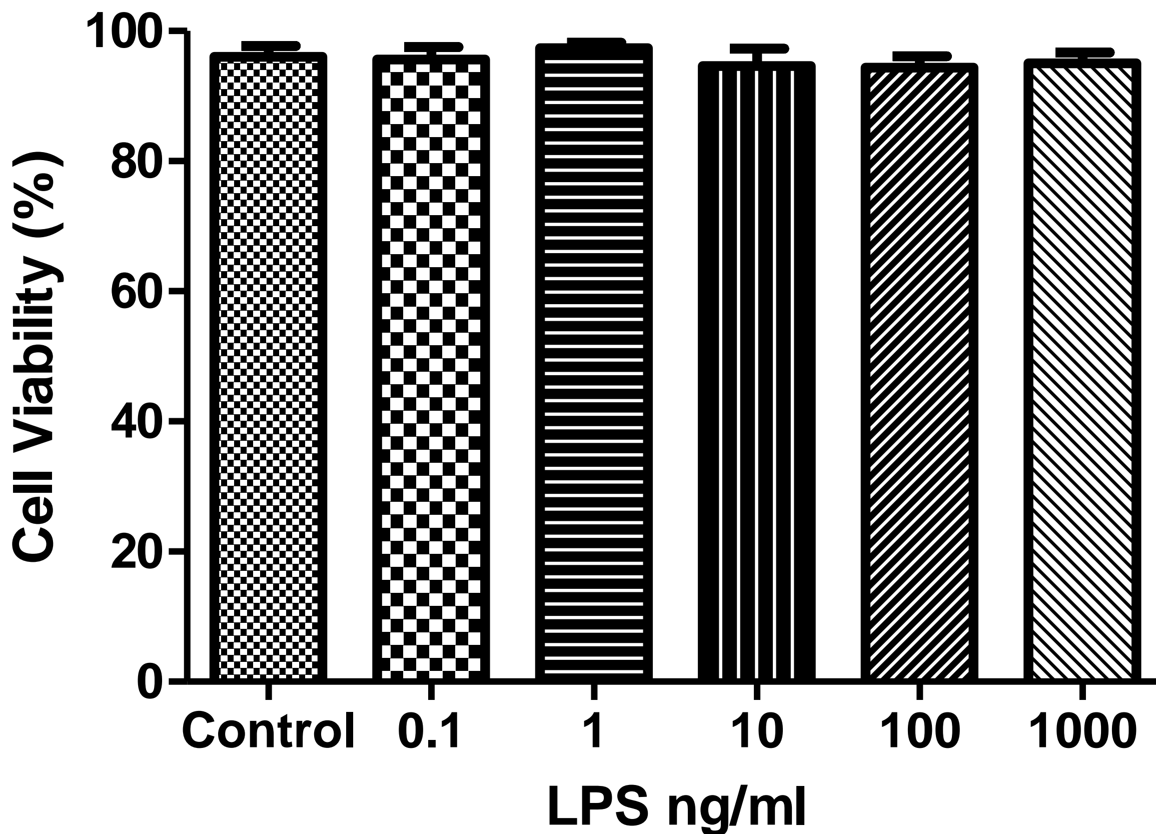
e. If HIV test is positive. What is the CD4 count results? ______________

9. Weight and Height

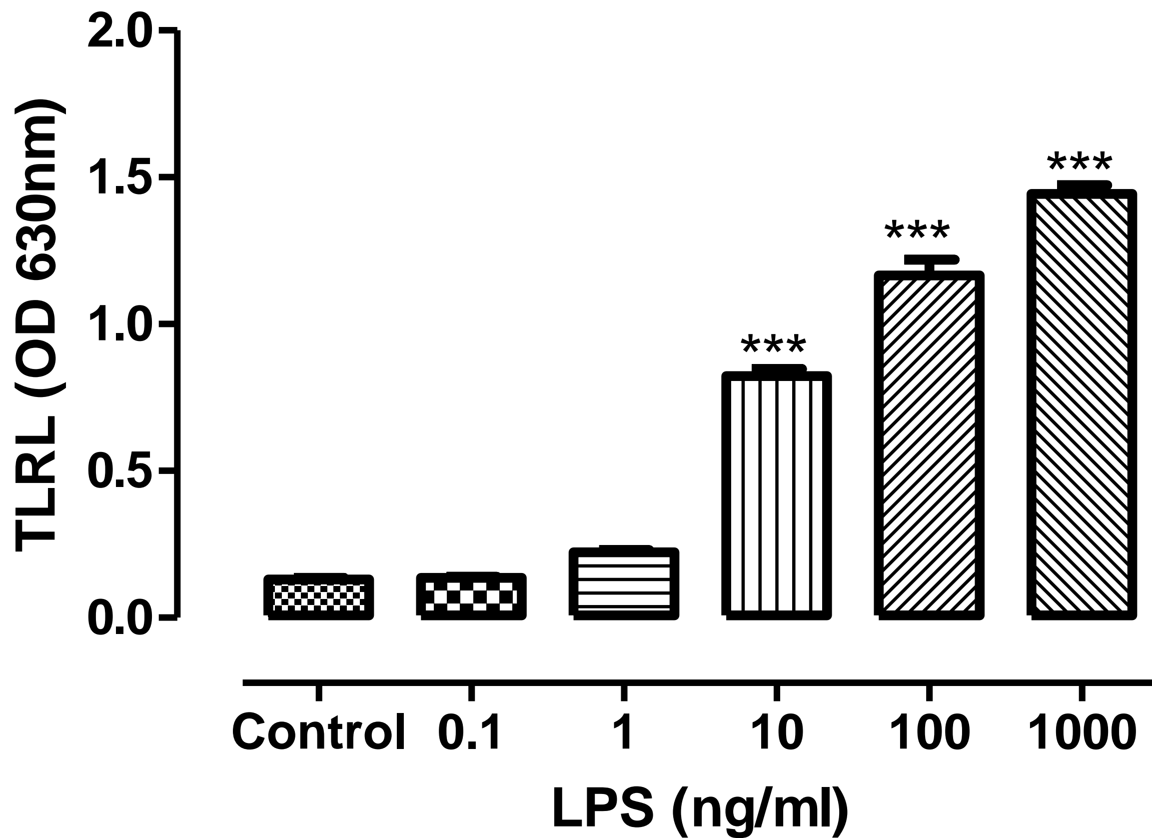
a. Measure and record height in cm _________________cm

b. Measure and record weight in kilograms _________________kg

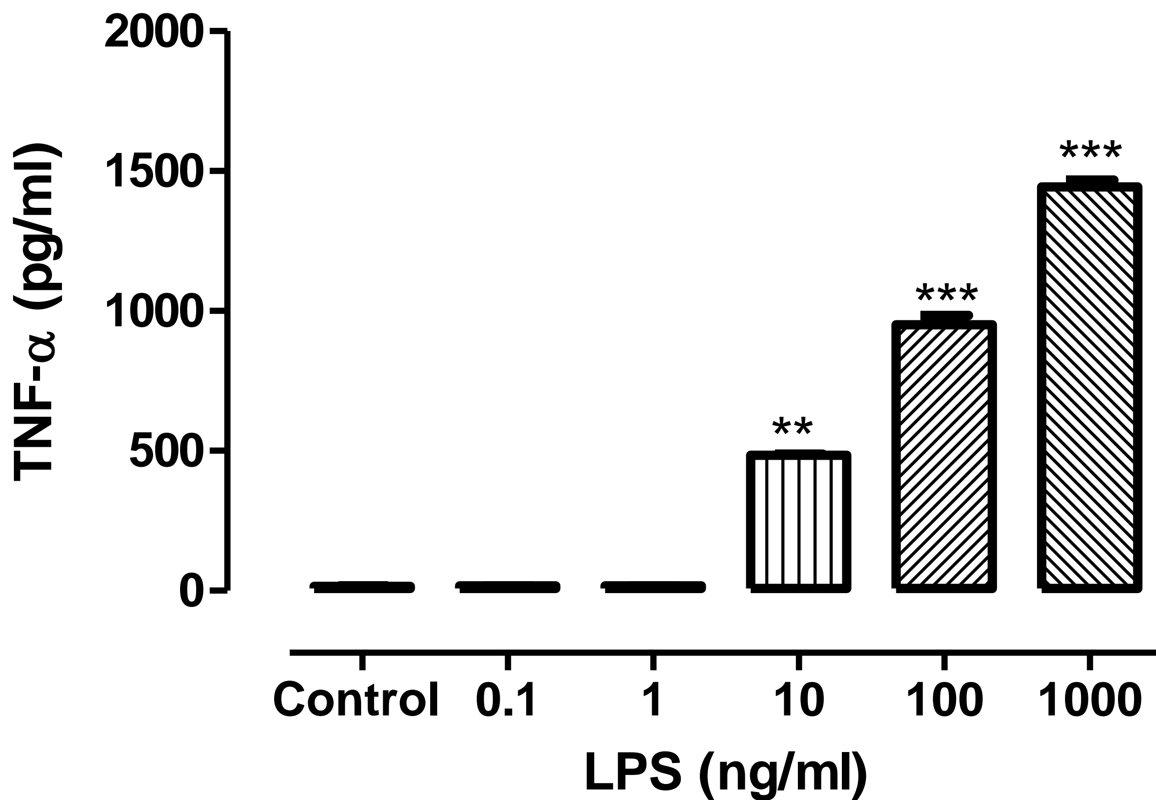
APPENDIX IX: Cell Culture Controls



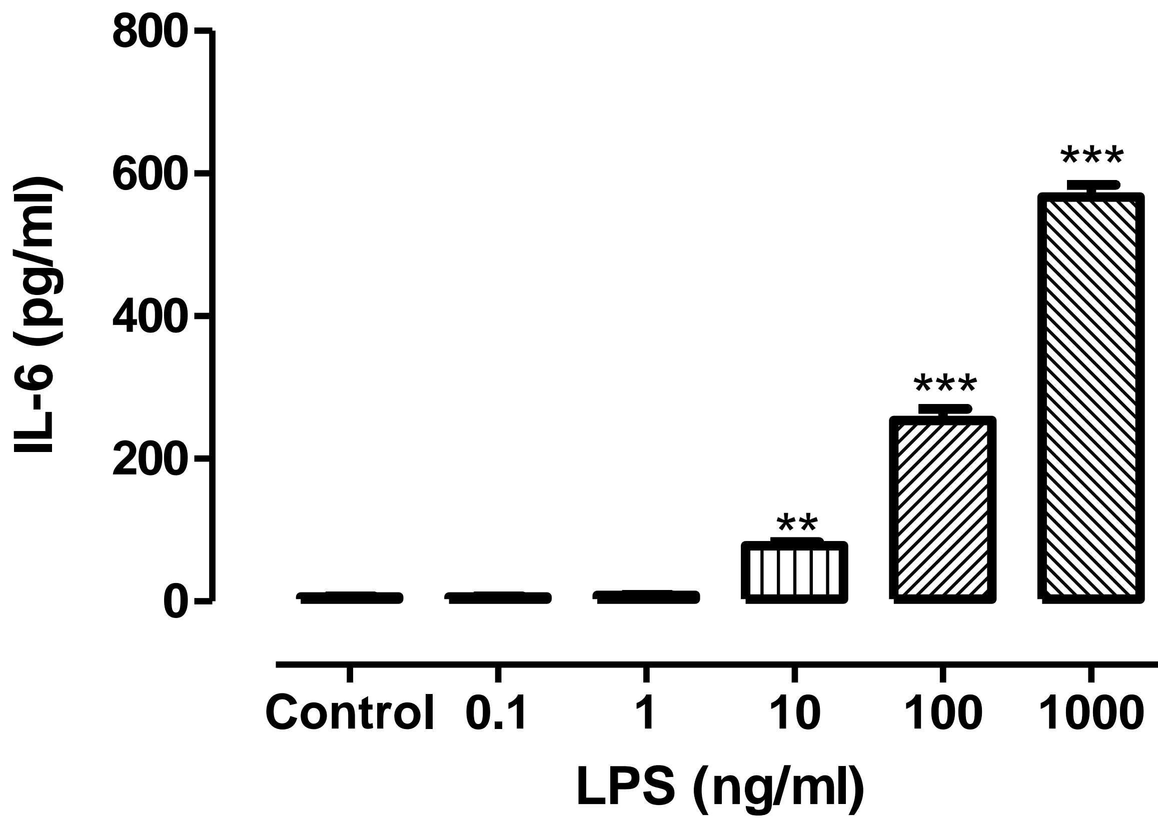
Effects of LPS on the viability of RAW-Blue cells treated with various doses of LPS for 24 hours. There was no statistical difference in cell viability at various concentrations and viability was more than 95%. Data are expressed as the means ± SEM (n=4) independent experiments compared with control.



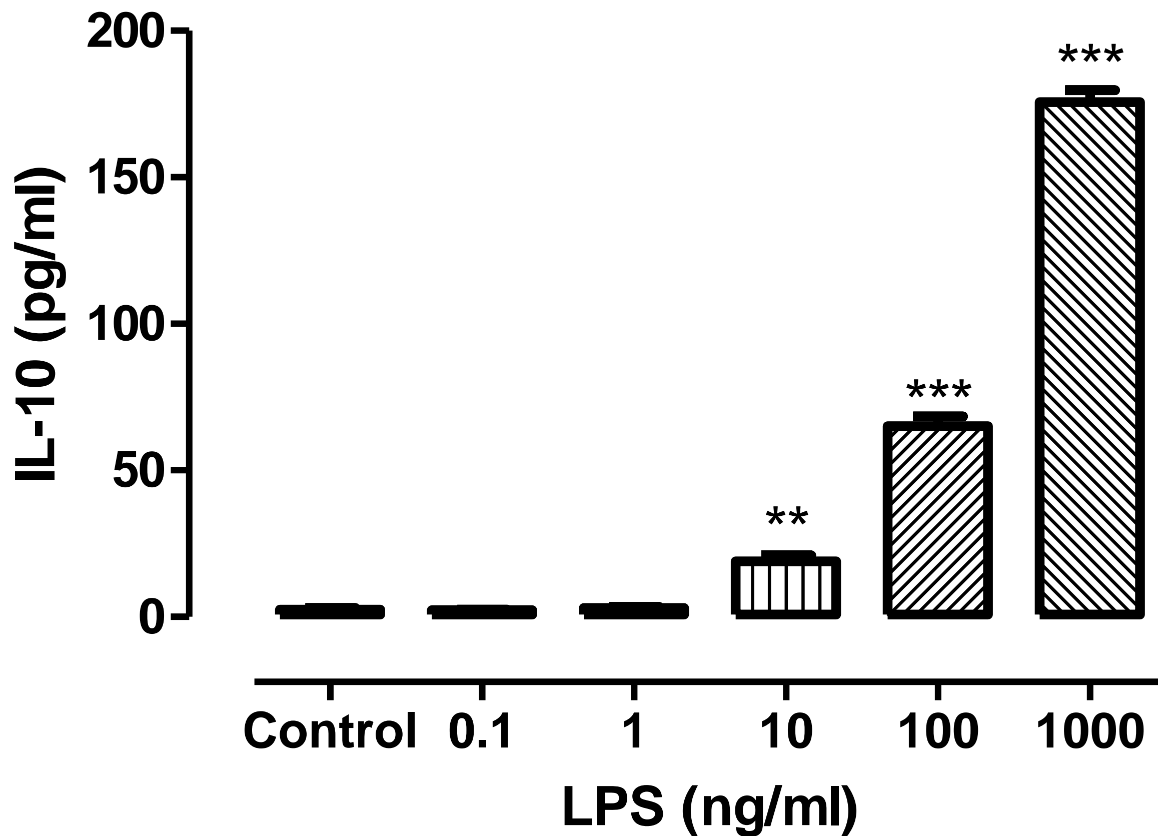
Production of (SEAP) TLR ligands activity after stimulation of RAW-Blue cells with various concentrations of LPS. There was production of TLRL activity at 10, 100 and 1000ng/ml. The data are representative of four independent experiments and expressed as mean ± SEM (n=4). ***p<0.001 compared with the control.



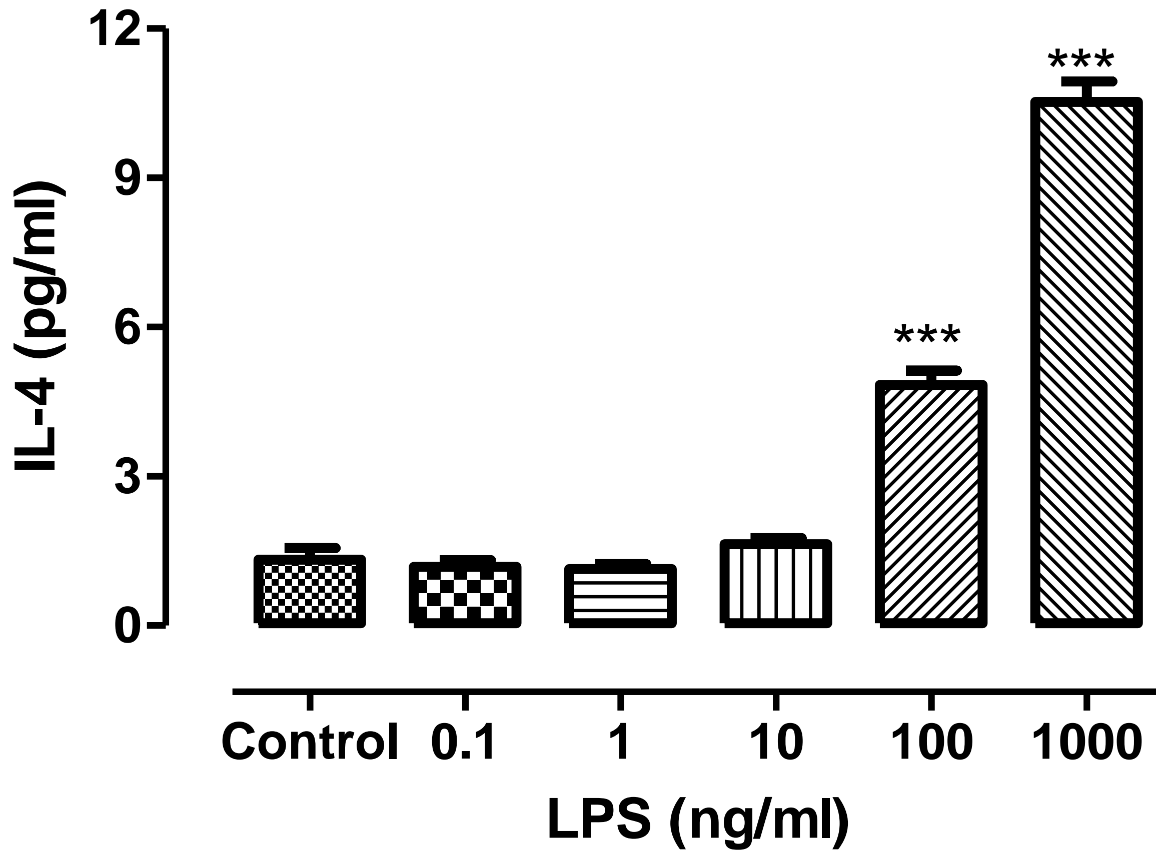
Production of TNF-α after stimulation of RAW-Blue cells with various concentrations of LPS. There was production of TNF-α at 10, 100 and 1000ng/ml concentrations of LPS. The data are representative of four independent experiments and expressed as mean ± SEM (n=4). ** p<0.01, ***p<0.001 compared to the control.



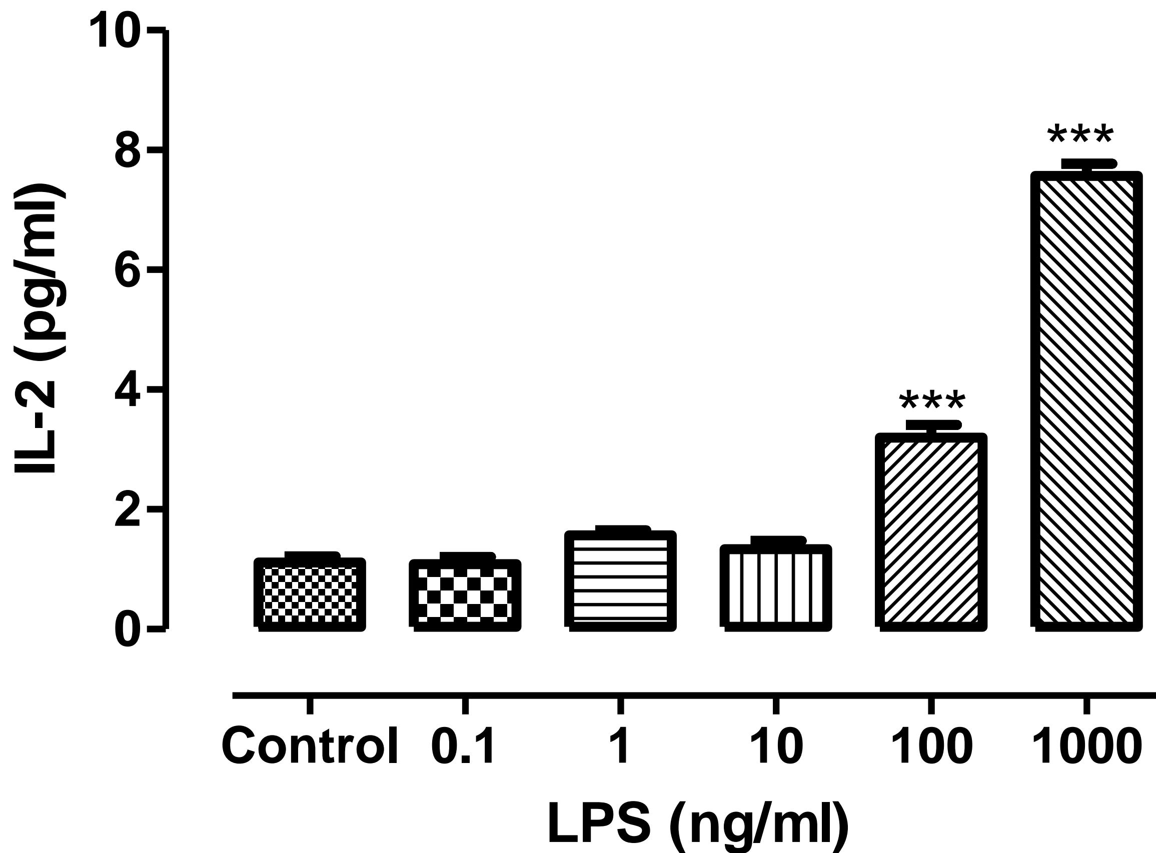
Production of IL-6 after stimulation of RAW-Blue cells with various concentrations of LPS. There was production of IL-6 at 10, 100 and 1000ng/ml concentrations of LPS. The data are representative of four independent experiments and expressed as mean ± SEM (n=4). ** p<0.01, ***p<0.001 compared to the control.



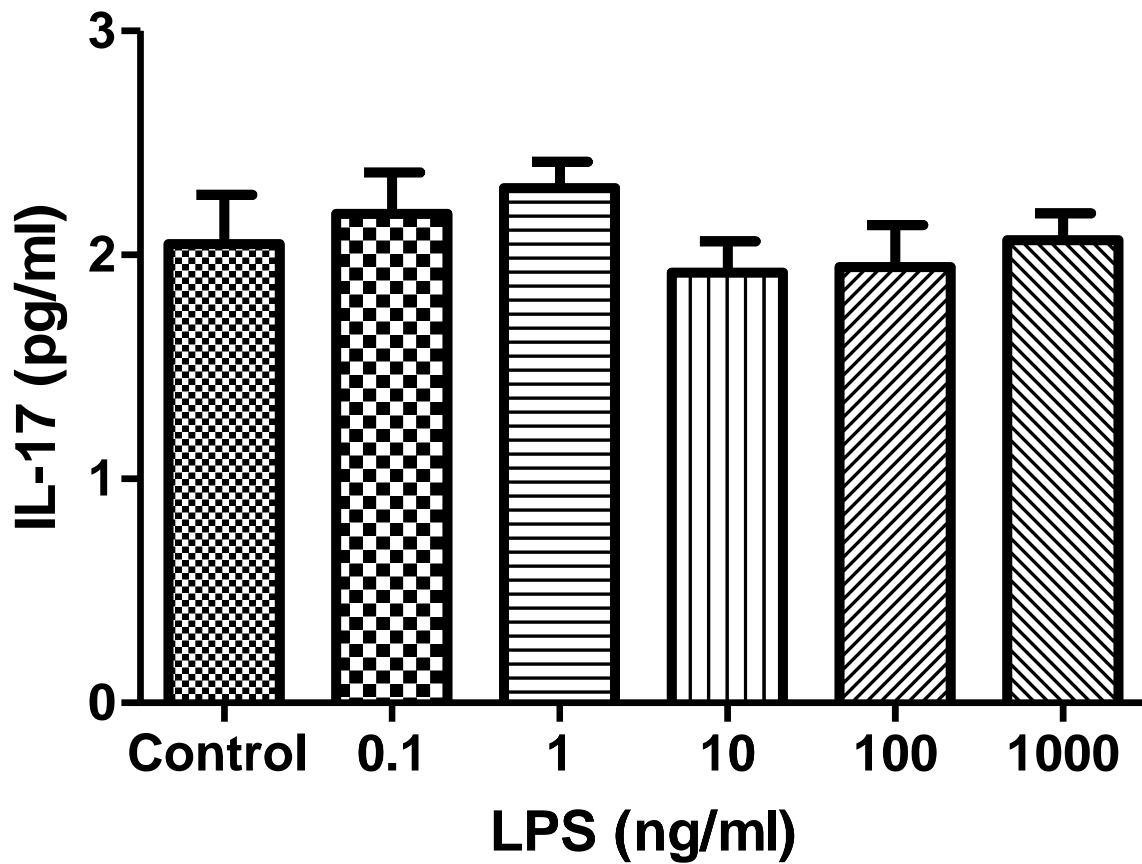
Production of IL-10 after stimulation of RAW-Blue cells with various concentrations of LPS. There was production of IL-10 at 10, 100 and 1000ng/ml of concentrations of LPS. The data are representative of four independent experiments and expressed as mean ± SEM (n=4). ** p<0.01, ***p<0.001 compared to the control.



Production of IL-4 after stimulation of RAW-Blue cells with various concentrations of LPS. There was production of IL-4 when cells were stimulated by 100 and 1000ng/ml concentrations of LPS. The data are representative of four independent experiments and expressed as means ± SEM (n=4). ***p<0.001 compared to the control.

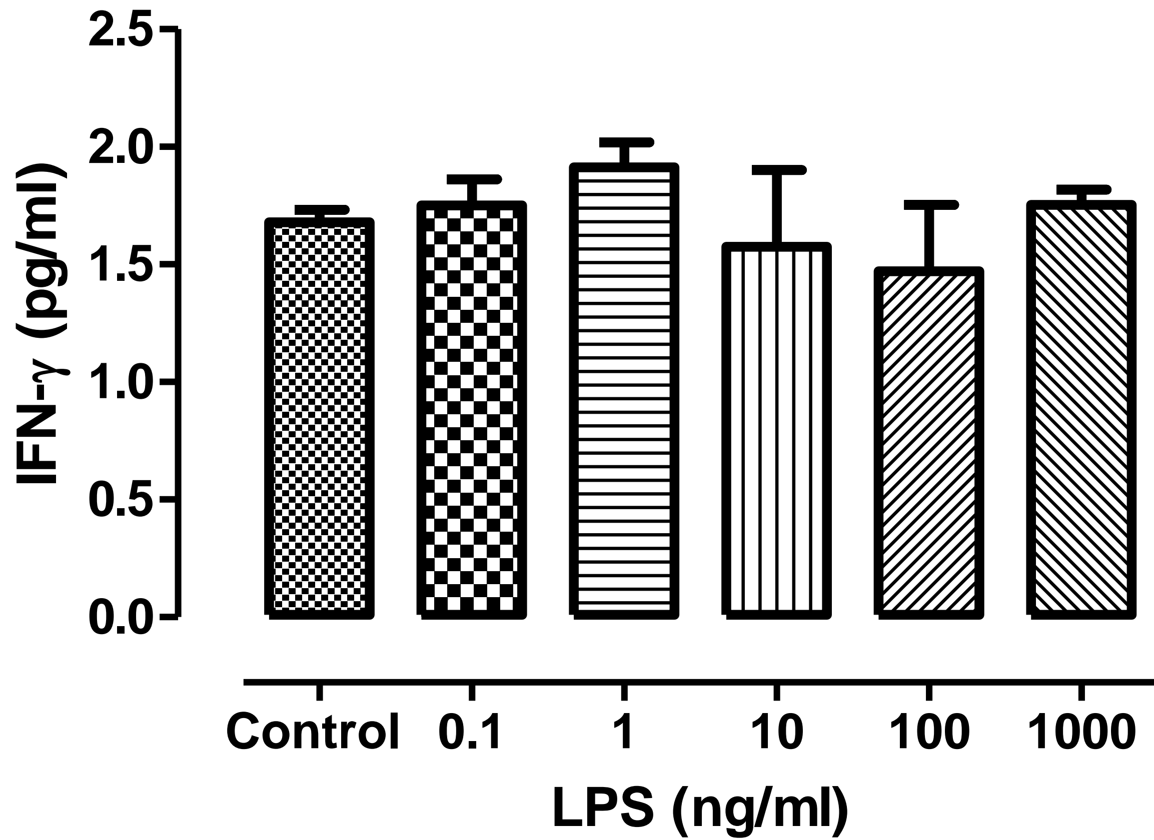


Production of IL-2 after stimulation of RAW-Blue cells with various concentrations of LPS. There was production of IL-2 when cells were stimulated with 100 and 1000 ng/ml LPS. The data are representative of four independent experiments and expressed as means ± SEM (n=4). ***p<0.001 compared to the control.



Production of IL-17A after stimulation of RAW-Blue cells with various concentrations of LPS. There was no statistical difference with the control in the production of IL-17 when cells were stimulated at different

concentration of LPS. The data are representative of four independent experiments and expressed as means ± SEM (n=4).



Production of IFN-γ after stimulation of RAW-Blue cells with various concentrations of LPS. There was no statistical difference with the control in the production of IFN-γ when the cells were stimulated with various concentrations of LPS. The data are representative of four independent experiments and expressed as mean ± SEM (n=4).