ASSESSMENT OF ENVIRONMENTAL ENTERIC DYSFUNCTION IN INFANTS IN MIRPUR, DHAKA, BANGLADESSH



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A Thesis

Submitted to

Khulna University, Khulna

In Partial Fulfillment of the Requirements for the Degree of

Master of Science in

Biotechnology and Genetic Engineering

By

Student ID: MS- 180709

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Khulna-9208

March, 2020

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Approved as to the style and content by

Dr. Md. Shamim Akhter
Supervisor
Professor
Biotechnology and Genetic Engineering-Discipline
Khulna University
Khulna-9208, Bangladesh

Dr. Rashidul Haque
Co-Supervisor
Senior Scientist and Head of Emerging Infections & Parasitology Laboratory.
International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B).

29.02.2022
Dr. Ayesha Ashraf
Chairman, Examination Committee
and
Head, Biotechnology and Genetic Engineering Discipline
Khulna University
Khulna-9208

March, 2020

DECLARATION

This thesis/report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor.



Rubel Howlader
Student ID: MS-180709
March, 2020

Dedicated

To

My Beloved Father and Mother

ACKNOWLEDGEMENT

At the first and foremost, I wish to express all of my devotion and reverence to the most merciful beneficent **creator** who has enabled me to perform this research work.

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The Author

March 10, 2020.

Abstract

Around 25 percent of the world's children aged < 5 have stunted growth, which is associated with increased mortality, cognitive dysfunction and productivity loss. Reducing the number of stunted children by 40 per cent is a global aim for 2030. An approximate 1 in 4 (23 per cent) children aged < 5 years have been stunted worldwide in 2017. With slow progress in stunting reduction in many regions and the realization that a large proportion of stunting is not solely due to insufficient diet or diarrhea, it remains that other factors have to explain the continued faltering growth. Environmental enteric dysfunction (EED), a subclinical state of intestinal inflammation, can occur in infants across the developing world and (EED) is an acquired enteropathy of the small intestine, characterized by enteric inflammation, villus blunting and decreased crypt-to-villus ratio. EED has been associated with poor outcomes, including chronic malnutrition (stunting), wasting and reduced vaccine efficacy among children living in low-resource settings. EED is proposed as an immediate causal factor connecting poor sanitation and stunting. Recurring exposure to fecal pathogens and changes in the composition of the intestinal microbiota are hypothesized to initiate this process which leads to a self-perpetuating cycle of pathology. We see EED as a primary gut disorder that drives chronic systemic inflammation, resulting in resistance to growth hormones and impaired linear growth. Mechanisms which contribute to EED growth failure include intestinal leakage and increased permeability, inflammation of the gut, dysbiosis and translocation of bacteria, systemic inflammation and malabsorption of nutrients; There is currently no agreed case description or EED gold standard biomarker, which challenges field studies. My goals are to define the evolution of EED during infancy, determine its contribution to stunting, identify associations between EED and biomarkers and also assess the relationship between EED and prospective linear Development at 52 weeks of infants.

Contents

Page no.

Acknowledgement	**I**
Abstract	**II**
Contents	**III-VI**
List of Figures and Tables	**III-VI**
Abbreviations and Acronyms	**vii-viii**
Chapter one: Introduction	**1-6**
Chapter Two: Review of Literature	**7-28**
Chapter Three: Materials and Methods	**29-44**
Chapter Four: Results and Analysis	**45-57**
Chapter Five: Discussion	**58-59**
Chapter Six: Conclusion	**60**
Chapter Seven: Bibliography	**61-67**

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List of Figure:

Chapter Two: Review of Literature **Page no.**

Figure 1: The intestinal epithelium in health (a) and with environmental enteric dysfunction (b). Adapted and reprinted with permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology.*	**07**
Figure 2. Hypothesized causal pathway to stunting through environmental enteric dysfunction.	**11**
Figure 3. Pathogenesis of EE/EED and long-lasting consequences.	**12**
Figure 4: Promotion of Immune Regulation by the Microbiota during Steady State and Inflammation (Left)	**13**
Figure 5: Promotion of Protective Immunity by the Microbiota:	**14**
Figure 6: The mucus represents the primary barrier limiting contact between the microbiota and host tissue preventing microbial translocation.	**15**
Figure 7: The lower gastrointestinal tract consists of the small and large intestines which are referred to as the gut.	**19**
Figure 8. Pathways of epithelial permeability	**20**
Figure 9. Overview of intestinal epithelial junctional complexes	**21**

Figure 10. Tight Junctions	**22**
Figure 11: Evaluation of intestinal permeability.	**23**
Figure 12: Three-dimensional structure of C-Reactive Protein	**24**
Figure 13: LPS interacts with LBP and CD14 to induce cell activation	**26**

Chapter three: Materials and Methods **Page no.**

Figure14: Lactulose	**29**
Figure15: Dionex ICS-5000 Ion Chromatography (IC) system Components in icddr,b Bangladesh.	**32**
Figure16: Schematic view of an HPIC instrumentation	**33**

Figure 17 Chromatogram of Lactulose Mannitol and Melibiose standard 1,2,3,4,5 and Chromatogram of urine specimen before and after lactulose mannitol solution intake	**34-37**
Figure19: ELISA KIT OF Soluble CD14	**42**
Figure20: Microplate reader used in calculating OD value.	**44**
Figure21: SPSS (version 10.5; SPSS Inc., Chicago, IL, USA) and WHO Anthro plus	**44**

Chapter Four: Results and Analysis **Page no.**

Figure 22: Level of three non-invasive biomarkers of nutritional status group.	**48**
Figure 23: Height for Age Z score and Lactulose -Mannitol Ratio	**53**
Figure 24: Height for Age Z score and Level of sCD14 in ng/ml	**54**
Figure 25: Height for Age Z score and Level of CRP in mg/ml	**55**
Figure 26: Level of sCD14 in ng/ml and Level of CRP in mg/ml	**56**

Figure 27: Level of sCD14 in ng/ml and Lactulose -Mannitol Ratio	**57**
Figure 28: Level of CRP in mg/ml and Lactulose -Mannitol Ratio	**58**
Figure 29: Level of three non-invasive biomarkers among male and female	**61**

List of Tables:

Chapter Four: Results and Analysis

Table 1: Male and Female percent	**45**
Table 2: Percent of Male and Female among Stunted, at risk of stunting and Healthy control	**45**
Table 3: EED by measuring L:M ratio at 52 weeks of children	**45**
Table 4: EED due to CRP at 52 weeks of children	**46**
Table5: EED due to sCD14 at 52 weeks of children	**46**

Table 6: Descriptive Analysis by Nutritional status Group	**47**
Table 7: Mann-Whitney Test for Stunted vs At risk of stunting	**49**
Table 8: Mann-Whitney Test for Stunted vs Healthy	**49**
Table 9: Mann-Whitney Test for Healthy vs At risk of stunting	**50**
Table 10: Descriptive Statistics of Four Parameters	**50**
Table 11: Correlations Among four parameters	
Table12: Group Statistics of four parameters between stunting and at risk of stunting	**59**
Table13: Group Statistics of four parameters between at risk of stunting and healthy control	**59**
Table14: Group Statistics of four parameters between at risk of stunting and healthy control	**60**

Abbreviations and Acronyms

AAT	**α-1 antitrypsin**
AGP	**α-1 acid glycoprotein**
AUC	**Area under the ROC curve**
BT	**bacterial translocation"**
CFS	**Complementary food supplement**
CHN	**Center for Human Nutrition (JHSPH)**
CP	**Chickpea CFS**
CRP	**C-reactive protein**
CV	**Coefficient of variation**
DAMPs	**danger-associated molecular patterns**
DDS	**Dietary diversity score**
EED	**Environmental enteric dysfunction**
ELISA	**Enzyme-linked immunosorbent assay**
EndoCAb	**Endotoxin core antibody**
F/V	**Fruits and vegetables**
GH	**Growth hormone**
GHRH	**Growth hormone releasing hormone**
GALT	**gut associated lymphoid tissue**
GLP-2	**Glucagon-like peptide-2**
HPIC	**High pressure ion chromatography**
HAZ	**Height for Age Z score**
icddr,b	**International Centre for Diarrhoeal Disease Research, Bangladesh**
IL-6	**Interleukin 6**
IgA	**Immunoglobulin A**
IGF	**Insulin-like growth factor**
IBD	**active inflammatory bowel disease**
IBS	**irritable bowel syndrome**
IGFBP	**IGF binding protein**
IgG	**Immunoglobulin G**
IS	**Inflammation score**
IUGR	**Intrauterine growth restriction**
JAMs	**junctional adhesion molecules**
Kcal	**Kilocalorie**
L:M	**Lactulose:mannitol ratio**
LAZ/HAZ	**Length/height-for-age z-score**
LC-PUFA	**Long-chain polyunsaturated fatty acid**
LM	**Lactulose mannitol solution**
LMIC	**Low- and middle-income country**
LPS	**lipopolysaccharide**

LSI	**Living standards index**
MDD	**Minimum dietary diversity**
MODS	multiple organ dysfunction syndrome
MPO	**Myeloperoxidase**
NEO	**Neopterin**
OPV	Oral poliovirus vaccines
PCA	**Principal component analysis**
PAMPs	**pathogen-associated molecular patterns**
PD	**Plumpy'doz CFS**
PLS	**Partial least squares regression**
sCD14	**Soluble cluster of differentiation 14**
SIBO	**Small intestine bacterial overgrowth.**
TNF	**Tumor necrosis factor**
TJs	**tight junctions"**
UNICEF	**United Nations Children's Fund**
USDA	**United States Department of Agriculture**
Vit.	**Vitamin**
WASH	**Water, sanitation and hygiene**
WAZ	**Weight-for-age z-score**
WFP	**World Food Programme**
WHO	**World Health Organization**
WLZ/WHZ	**Weight-for-length/height z-score**



entity will still be eliminated soon, as efforts have been inadequate to implement WaSH at the highest coverage(14). For example, it is estimated that at least 1.8 billion people worldwide drink fecally contaminated water, and only 68% have access to "improved" sanitation facilities as interpreted by the Joint Monitoring Program of the United Nations (22).

Additionally, the capacity to implement hygiene practices is constrained due to limited access to water of any quality. Consequently, even if WaSH can deter EED to some extent, many children will still continue to suffer from EED and its consequences and there is considerable interest in the detection and testing of therapeutics (29). Due to its obscure and largely sub-clinical etiology, no clear role for a single candidate biomarker has been established.

Consequently, over the past decade, there has been a gradual increase in interest to study the role of altered small bowel histology and function in the pathogenesis of stunting and malnutrition in early childhood (15). This is the result of several factors, including the continuing prevalence of stunting in young infants and children in developing countries (16). Strategies to study this altered nutritional status have included numerous nutritional and healthcare interventions(3, 4) and the introduction of new methods for assessing intestinal function suitable for use in this age group(17, 18). Some preliminary evidences indicate that the permeability of small intestines to sugar substrates is in at least a few pioneering studies, inversely correlated with growth(in both weight and height) (19, 20)in early postnatal life. These studies not only noted the frequency of measurable absorptive intestinal dy sfunction, but also provided evidence for a systemic and intestinal inflammatory response as an additional (21, 22) potential contributor to growth failure and a potential target for interventions to interrupt its causative pathways(23).
Early studies recorded morphometric changes in South Asian residents ' small intestine mucosa (9, 10), findings supported by more recent confocal microscopy data (11), routine endoscopic examination among EED-risk children is assumed unreasonable because of their value, invasiveness, and need for technical expertise. Many researchers therefore have pursued less invasive EED measures, includin g gastrointestinal permeability tests, absorption, enterocyte mass and function. Given the wealth of evidence demonstrating the integral role of EED in child health, development, and growth, there is currently no single gold standard invasive diagnostic test to identify EED.
Although The efficiency of intestinal absorption tests and evidence of increased permeability of the intestinal mucosa,(22) evaluated by the dual sugar absorption test of lactulose: mannitol (or rhamnose, (17, 18) was recently examined by Prendergast and Kelly ((24)).In this study, the authors indicate that such enteropathy can play a major role in the linear growth retardation that is so common in developing-country settings among young infants. A better understanding of the causes and natural history of this enteropathy can lead to effective interventions being developed, significantly reducing the burden of onset of early childhood malnutrition, reducing susceptibility to recurrent infections, promoting growth and development, and improving child mortality in developing countries (3, 21).

Undernutrition results most commonly from limited quality or quantity of food, suboptimal feeding practices, and high rates of infectious diseases (6,7). Wasting is usually considered to be a short-term (i.e., acute) response to inadequate intake or an infectious disease episode, whereas stunting is considered to be a longer-term response to a sustained poor dietary intake or repeated illnesses. If linear growth slows over the short term, catch-up growth may occur

once the infection is resolved or if diet improves (8,10). However, in many resource-poor settings of low- and middle-income countries, dietary intake is consistently
inadequate and infectious diseases are common, impeding the process of ca tch-up growth and potentially resulting in many stunted children. Associations between stunting and wasting in children are not consistently found in analyses using cross-sectional data (11–14), likely because wasting is a short-term and potentially seasonal phenomenon resulting from a recent insult (infection or food insecurity), whereas stunting results from a longer-term multifactorial process of undernutrition. In addition, wasting may precede linear growth retardation (15); therefore, cross-sectional data may not demonstrate a concurrent relationship.
Using a large longitudinal dataset, to explore the longitudinal relationship between stunting and wasting. Specifically, if the history of wasting instances is related to stunting at 18–24 mo. of age. By increasing our understanding of these relationships, we can develop better interventions that will improve child health and survival in developing countries.

Lactulose: mannitol (LM) test:

A number of tests based on the passive absorption of simple sugars have been used to assess gut permeability as an index of recovery from inflammatory bowel disease (IBD) (30, 31) and from autoimmune diseases such as coeliac disease. The ratio of the quantities of urinary lactulose and mannitol excreted during a given period has been used most frequently (32), although a number of workers have used rhamnose rather than mannitol. All three sample sugars are ingested passively from the intestine, not metabolized extensively, and excreted unchanged in urine in proportion to the amounts absorbed (33).

The lactulose: mannitol (LM) test is a quantitative experimental procedure to directly measure the potential of two non-metabolized molecules of sugar — lactulose and mannitol — to permeate the mucosa of the intestine. (34, 35). Mannitol, a readily absorbed monomer, functions as a marker for transcellular absorption and lactulose, a dimer, is only partially absorbed and serves as a marker for the integrity of the mucosa. An elevated mannitol -to-lactulose ratio is an indicator of dysfunction of the intestinal barrier. (35, 36).The LM test can be used as a non-invasive diagnostic aid to detect dysfunction of the intestinal barrier in patients with diarrheal diseases, malnutrition, HIV infection, trauma, surgical stress, celiac dis ease, Crohn's disease, active inflammatory intestinal disease (IBD), irritable intestinal syndrome (IBS), cow's allergy and intolerance, lactase deficiency, chemotherapy -induced mucositis or chemotherapy-induced mucositis(35, 37).

Given the enormous potential of the test, clinical research implementation of the test remai ns limited due to variability in methodologies such as: population study, administration of sugar solutions, time to collect urine, and method of assay between studies. In addition, after consumption of sugar solution, urine is collected for 5 to 6 hours; this longer processing time makes the experiment somewhat inconsistent.
The ratio of the quantities of urinary lactulose and mannitol excreted during a given period has been used most frequently, although a number of workers have used rhamnose rather than mannitol. All three probe sugars are passively absorbed from the intestine, not extensively metabolised, and excreted unchanged in urine in proportion to the quantities absorbed[9] In order to compensate for any variations in the surface area of the inte stinal mucosa between participants, the division of the quantity of lactulose excreted by the quantity of mannitol

excreted i.e. the use of ratios was postulated. Subsequent work has however demonstrated a number of factors that may confound the basis of such comparisons as they render the result dependent upon the type of sugar used and the time period over which the cumulative urinary excretion was determined. Firstly, the rate of excretion of the two 'reference' sugars that are assumed to reflect surface area i.e. mannitol or rhamnose, A tendency due to villus atrophy, is significantly reduced in a variety of inflammatory conditions. Second, the average excretion levels of both reference sugars vary, because mannitol is significantly higher than rhamnose. Thirdly, the lactulose and mannitol excretion levels vary over time from dosage due to the differing permeabilities of the small intestine and colon to these sugars These differences cause the ratio of the quantities of the two sugars to vary with the period of time over which their excretion is determined. Thus, the early peak in mannitol excretion causes the LMR to be lower when cumulative excretion is assessed over 2 hours whilst the later peak in lactulose excretion and lower rate of mannitol excretion 4 hours after dosage will cause the LMR to increase when cumulative excretion is assessed over 4 or more hours. These effects may be magnified in the presence of a pro-inflammatory condition or a proinflammatory stimulus such as a single 600 mg of aspirin as these will lead to an increase in the overall excretion of lactulose and a decrease in mannitol. These different problems have led researchers to propose that temporal absorption profiles of the two sugars should be considered separately and in a ratio.

C-reactive protein (CRP):

C-reactive protein (CRP) is an acute-phase protein that is released upon infection or inflammation, with its levels rising up to 1000 fold at sites of inflammation. CRP is produced as a homopentameric protein, termed native CRP (nCRP), which can irreversibly dissociate at sites of inflammation and infection into five separate monomers, termed monomeric CRP (mCRP). CRP is synthesized primarily by hepatocytes, though there are reports of CRP being synthesized by smooth muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes. Evidence suggest that estrogen in the form of hormone replacement therapy influences CRP levels in the elderly. Traditionally used as a marker for infection and cardiovascular events, there is now growing evidence that CRP plays a major role in inflammatory processes and host responses to infection, including complementary pathway, apoptosis, phagocytosis, release of nitric oxide (NO) and cytokine production, particularly interleukin-6 and tumour necrosis factor-α.

C-reactive protein (CRP) is an annular (ring-shaped) protein found in blood plasma, the circulating concentrations of which increase as a response to inflammation. It is an acute-phase protein of hepatic origin, which increases by macrophages and T cells after interleukin-6 secretion. Its physiological role is to bind to lysophosphatidylcholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via C1q. (5) CRP is synthesized by the liver in response to factors released by macrophages and fat cells (adipocytes).

In response to factors released by macrophages and fat cells (adipocytes), CRP is synthesized by the liver. It is a member of the protein family of pentraxins. It is not related to C-peptide (insulin) or protein C (blood coagulation). The first pattern recognition receptor (PRR) to be identified was c-reactive protein.

CRP binds to the phosphocholine expressed on the surface of dead or dying cells and some bacteria. This activates the complement system, promoting phagocytosis by macrophages, which clears necrotic and apoptotic cells and bacteria.(13) This so-called acute phase response occurs as a result of increasing concentrations of IL-6, which is produced by macrophages(6) as well as adipocytes(7) in response to a wide range of acute and chronic inflammatory conditions such as bacterial, viral, or fungal infections; rheumatic and other inflammatory diseases; malignancy; and tissue injury and necrosis. These conditions cause interleukin-6 and other cytokines to be released which trigger liver synthesis of CRP and fibrinogen.

CRP attaches the micro-organisms to phosphocholine and It is thought to assist in complementing attachment to foreign and damaged cells, and to promote macrophage phagocytosis (opsonin-mediated phagocytosis), which expresses a CRP receptor. As an early Défense system against pathogens, it plays a role in innate immunity (3).

Soluble CD14:

CD14 is a 55-kD protein found both as a glycosylphosphatidylinositol-linked protein on the surface of mononuclear phagocytes and as a soluble protein in the blood. CD14 on the cell membrane (mCD14) has been shown to serve as a receptor for LPS-binding protein lipopolysaccharide complexes but a soluble CD14 (sCD14) role has not been described.
Soluble CD14 in the HIV epidemic is associated with morbidity and mortality. It is a lipopolysaccharide (LPS) co-receptor that is released upon activation from the monocytes (25). Here we demonstrate that inflammatory cytokines can trigger the release of sCD14 from healthy donors in peripheral blood mononuclear cell cultures, and that TLR ligands other than LPS can cause a reduction in CD14's monocyte cell surface expression. As such, sCD14 is a monocyte activation marker, not limited to LPS activation (26).

For HIV-infected patients, elevated plasma levels of soluble CD14 (sCD14) are associated with poor prognosis, and are a strong predictor of morbidity and mortality. Increased plasma sCD14 levels can continue even in patients treated with HIV infection, with long-lasting viremia control, and this is correlated with diminished CD4 + T-cell restoration(10). Additionally, sCD14 plasma levels were correlated with cardiovascular disease in both HIV infection and HIV-uninfected patients. In pediatric inflammatory lung diseases, in chronic hepatitis C (HCV) and hepatitis B (HBV) infections, elevated plasma levels of sCD14 are also reported, and in rheumatoid arthritis.
CD14 is a myeloid differentiation marker found primarily on monocytes and macrophages, although the neutrophils also show low rates(27). CD14 can exist as a GPI-anchored membrane protein or as one of two soluble isoforms that can be generated either by cleavage from the surface of the cell or released from intracellular pools(28). CD14 is a co-receptor for lipopolysaccharide (LPS) –a cell wall component of gram-negative bacteria –and it has been suggested that high levels of sCD14 in the plasma are reflective of LPS exposure(29).
The origin of the distribution of the soluble CD14 (sCD14) is unknown. To investigate whether CD14 could be an acute-phase protein (APP), ELISA determined the levels of sCD14, IL-6, and C-reactive protein in the serum and synovial fluid (SF) of patients with different arthropathies, and investigated the regulation of CD14 synthesis in liver cells. In patients with crystal-mediated or immunologically mediated arthritis (rheumatoid arthritis), serum levels of sCD14 were higher than or similar to those found in infection-mediated arthritis (reactive

arthritis), precluding a relation with bacteria exposure. Levels of sCD14 were similar in SF and serum, and did not correlate with the number of SF Not associated with the number of SF leukocytes by protease-mediated shedding, excluding an important source of leukocyte membrane bound CD14. Conversely, sCD14 serum levels in patients correlated with those of C-reactive protein, a classical APP, andIL-6, a cytokine known to regulate APP synthesis in the liver. The serum sCD14 levels have associated with disease behaviour in patients with rheumatoid arthritis and reactive arthritis. has activated hepG2 hepatoma cells to produce CD14. The inducibility of CD14 via IL-6 was also observed at the mRNA level in both HepG2 cells and primary human hepatocytes by real-time PCR. These in vitro findings have been supported by in vivo experiments in IL-6/mice injected with turpentine, an acute-phase reaction experimental model. CD14 mRNA liver levels increased in IL-6 range / range but not in IL-6 range / range mice. These findings indicate that sCD14 can be considered an APP of type 2.

Finally, the Hippocratic quote "all disease begins in the gut" seems to be true, over 2,000 years later, for diverse pathological conditions. Our knowledge on the pivotal role of the intestinal barrier and gut microbiota in health and disease has been majorly developed and constitutes currently a scientific field of intense research. Clinicians should not neglect considering this central pathophysiological role of the gut and should apply all necessary preventive measures to protect the integrity of their patients' intestines in diverse intestinal and extra-intestinal diseases. Future research with application of modern systems biology approaches, namely using genomics, transcriptomics and proteomics might lead to specific and potentially individualized pharmacological targets for intervention to control intestinal hyperpermeability.

Chapter Two: Review of Literature

Background History:

The healthy small intestine is a complex organ consisting of multiple functional elements: a mucus layer containing defensins and immunoglobulins,(38-40) a single layer of epithelial cells sealed by tight junctions, and the lamina propria and submucosa containing immune cells(41) .The intestinal epithelium, illustrated in forms a one-cell-thick interface between the internal organism and external luminal environment. It also comprises two major compartments, the villus and the crypt, which play major roles in the digestion and absorption of nutrients, in absorption and secretion of water and electrolytes and in intestinal immune function (42). The villous compartment comprises mature, absorptive cells that form finger-like projections extending into the intestinal lumen thereby amplifying the absorptive surface area 20-fold(40, 43). The crypt is a contiguous pocket of epithelial cells at the base of the villus that is populated by younger epithelial cells involved primarily in secretion of antimicrobial proteins and stem cells that continually divide (44)and migrate towards the villous surface. Collectively,(45) these features are responsible for the barrier,(46) immune and absorptive functions of the small intestine.(47)

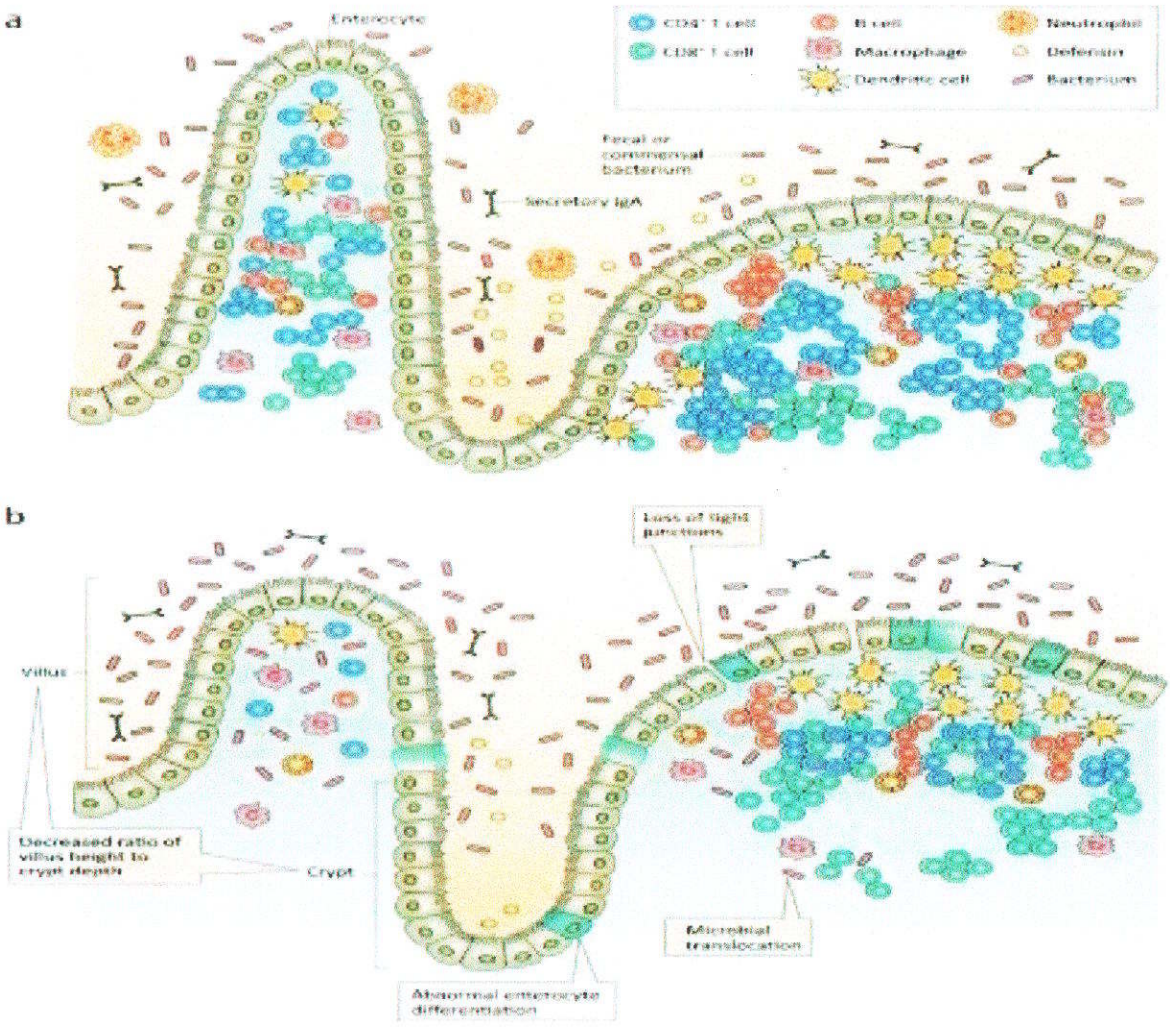


Figure 1: The intestinal epithelium in health (a) and with environmental enteric dysfunction (b). Adapted and reprinted with permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology.*

Environmental enteropathy / Environmental Enteric Dysfunction (EE / EED) was first described in the 1960s as "tropical enteropathy" (48, 49)and was defined by chronic histological changes in small intestinal inflammation in tropical (50). Histopathological abnormalities were characterized by decreased villous height, crypt hyperplasia, lamina propria lymphocytic infiltration,(51, 52) and increased intraepithelial lymphocytes, accompanied by abnormalities in the absorptive function of the small intestine, such as xylose and vitamin B12(53). Since its discovery, tropical enteropathy had not been recognized as a priority health issue because it does not cause overt symptoms (54)and was seen in apparently healthy individuals. However, EE / EED has risen in prominence as a potential cause of growth faltering in children living in developing countries(55, 56). Also, recent studies demonstrated the association of ED / EED with poor neurocognitive development and low vaccine efficacy resulting in the current great concern of ED / EED in public health of developing countries (57).

Objective of the study:
General Objective:

1. Association between intestinal infection and EED (Environmental Enteric Dysfunction).
2. Describe risk factors for EED by evaluating biomarkers.
3. Identification of intestinal permeability by evaluating lactulose mannitol ratio (L:M), CRP (C-reactive protein) and Soluble CD14
4. Prevalence of EED among male and female.

Specific Objective:

1. To identify correlation between EED and Biomarkers.
2. To assess relationship between EED and prospective Linear Growth.

Normal Gut Physiology

The healthy small intestine is responsible for digestion, absorption, barrier function and local immune responses, a diverse and critical set of interrelated functions essential for healthy growth. Digestion of foods consumed occurs throughout the stomach and small and large intestines, but the majority takes place in the small intestine(58). The length and structure of the small intestine allows for maximal absorptive capacity, with repeated folds covered in protruding villi themselves covered in a "brush border" of microvilli serving to increase the absorptive capacity through expansion of the surface area(59).The brush border is also a site of enzyme secretion and digestive activity that further facilitates nutrient absorption. In addition to absorbing nutrients required for maintenance and growth, the small intestine also resorbs vast quantities of water(60) and digestive enzymes excreted to aid in digestion and resorbed for repeated use(61).

Immune capabilities develop throughout infancy, primed by immune components in breast milk and habitual exposure to benign and pathogenic foreign particles (56, 62) The wall of the small intestine has its own local immune system that identifies and clears pathogens (43, 63). It also has the capability to present pathogens into the lymph system to initiate a systemic immune

response when needed(64, 65). This triage capacity serves to modulate the severity of immune response, as the gut becomes habituated to its perpetual contact with relatively harmless foreign matter. Emerging evidence suggests that the intestinal microbiota play a critical role in priming the immune system to respond appropriately to pathogenic exposures (66, 67)When the barrier function of the small intestine is compromised, pathogens may circumvent this local immune system and enter the bloodstream directly, triggering more frequent systemic immune responses.

EED Physiology

In the transition from theory to application, a detailed case definition of EED was never developed. Initially, this condition of growth-inhibiting enteropathy was described as a residual state of intestinal inflammation and increased permeability following an episode of diarrheal illness(2, 68) More recently, EED, as the condition has come to be termed, has been treated as the presence of said inflammation and permeability in the absence of acute diarrheal illness, irrespective of recent morbidity history(68). Still, some researchers focusing on intestinal permeability during the recovery from acute or persistent diarrhea continue to use the term "EED", further confusing the case definition of the condition(69) .While a precise definition for diagnostic purposes does not exist at present, researchers generally agree that the condition is characterized by crypt hyperplasia and partial villous atrophy, as well as leakiness in the tight junctions between enterocytes. In the healthy gut wall, villi protrude i nto the intestinal lumen, increasing the surface area available for absorption, while the enterocytes of properly functioning crypt regions produce enzymes necessary for nutrient digestion and absorption. Crypt hyperplasia and villous atrophy cause a reduc tion in the surface area of the small intestine available for secretion of digestive enzymes and absorption of nutrients,which may reduce total nutrient absorption to below physiological requirements. At the same time, the tight junctions between enterocytes maintain the barrier between the digestive tract and the bloodstream, preventing microbial translocation and allowing trans-cellular mechanisms to regulate nutrient absorption. When leaky tight junctions allow for translocation of pathogens into the bloodstream, a systemic immune response may follow, which is hypothesized to explain the elevated acute phase proteins commonly observed in children with EED but no acute illness.93 Persistent low-grade systemic inflammation of this nature could be another important mechanism whereby EED inhibits growth.

Burden and distribution

Environmental enteric dysfunction is prevalent in contexts with poor sanitation facilities and hygiene practices, and is thought to result from repeated gastrointestinal exposure to environmental pathogens and toxins.(68, 69) These conditions abound in LMICs, as discussed above with respect to causes of stunting. The majority of trials that have reported the proportion of children with impaired gut integrity (generally elevated L:M ratio) compared to an age-matched healthy developed country population have found the prevalence of EED to be upwards of 80%,(70, 71) though those estimates are highly dependent on the cutoff used for "normal" L:M ratio, which ranges widely in the literature despite minimal reference to detailed normative data. Reported rates of EED are even higher in children hospitalized with acute malnutrition.

A loosely defined idiopathic subclinical intestinal condition common in tropical settings and distinct from symptomatic tropical sprue has been described for some time. Interest in subclinical enteropathy as a cause of stunting in children surged in the early 1990s, spurred by

research from The Gambia suggesting that elevated L:M ratios were highly prevalent and closely related to stunting in that setting. Participating infants had elevated L:M values at 76% of monthly assessments and the authors reported that 40% of observed stunting was explained by L:M. That research group continued to characterize EED using L:M ratio at the MRC Dunn Nutritional Laboratory in The Gambia, describing its burden across age groups in the population, histological characteristics of the condition and relationships with nutritional status and infection history. That ongoing work largely supported their initial conclusions, that EED is highly prevalent in the population, associated with inflammation and stunting in early childhood and of ambiguous origin.

At the same time, researchers investigating stunting in other settings began to undertake studies of enteropathy in their populations, producing a flurry of reports on the prevalence and risk factors for the condition, based largely on L:M ratios, in their specific populations. (For example, studies in Sao Paulo, Brazil; Guatemala; Malawi; Bangladesh; and Nepal.) While each report of prevalent EED spurred further EED studies, divergent assessment methods, age groups, inclusion criteria and other study design considerations make aggregating findings across these numerous studies challenging.

Epidemiology and Etiology of EED

Initially described as a condition of the tropics enteropathy (14)is virtually ubiquitous among persons living in conditions of poverty. South Asian adults with this condition and Peace Corps volunteers who had spent some time in such environments were observed to 'recover' when they spent a duration of time away suggesting that the causative factor was environmental (72, 73). However, recovery rates between these groups differ: South Asian adults who migrated to Europe or the United States had enteropathy on arrival, which resolved within ~5 years (while asymptomatic American soldiers in Vietnam and Peace Corps volunteers in Pakistan) who acquired enteropathy within a few month's residence in these settings recovered in the course of 4–5 months after returning to the United States(13, 74). This suggests that enteropathy is reversible, but that recovery is relatively slow, especially among people who have lived in an unsanitary environment and presumably had the condition throughout their lifetime.

Although the pathogenesis of EED is unclear (75), it has been linked to environmental contamination in general and faecal contamination in particular and likely represents an adaptive response to a contaminated environment (76). In response to a prolonged and persistent exposure to enteric pathogens and enterotoxins expressed by pathogenic bacteria, intestinal morphology is altered in a number of ways; the most frequently observed being crypt hyperplasia and villous atrophy (14). In EED, crypts are elongated with rapidly increased cell production rate This crypt hyperplasia is etiologically related to partial villous or shortening, fusing and broadening of the villi – resulting in the architecture of the gut becoming flatter and blunted with an appearance of leaves and ridges rather than the typical finger like (72). At the cellular level, hyperstimulation of enteric T-cells appears to be important in the pathogenesis of EED (73).In a human fetal intestinal explant model, a cell-mediated immune response was elicited by stimulating T-cells resulting in a 10-fold increase in the rate of crypt epithelial cell proliferation and subsequently shorter villi, demonstrating that activation of T-cells is important in the pathogenesis of EED(77, 78) and that crypt hyperplasia precedes villous atrophy(79) .EED can also be acquired by a certain period of time of living in poor sanitation

and hygiene(46), whereas celiac disease, showing the similarities with EE / EED histologically(45), occurs among genetically susceptible individuals exposed to gluten containing foods. Early investigations of Pakistanis and visiting residents to Pakistan showed that differences in intestinal permeability and absorptive capacity were related to the area of residence, not the areas of their origin(80).

Definition of EED and the difficulties in diagnosis in children:

Identification of blunted villi and crypt hyperplasia in biopsy specimens obtained via upper gastrointestinal endoscopy is the current gold standard for EE / EED diagnosis(81). However, these histological changes, despite the various extents in each individual(82), are seen in almost all people living in unsanitary conditions in developing countries), whereas extent of poor physical development varies widely(45). Taken together, the presence of biopsy abnormalities may not be a predictor of poor EE / EED outcomes, such as faltering growth, oral vaccine failure and poor neurocognitive development. In addition, poor growth is often apparent within 1–2 years and is largely irreversible (83).It is also known that undernutrition (lower height and weight) at 2 years is associated with lower human capital, repres ented by shorter adult height, less schooling, Lower economic efficiency and weight of offspring from multiple prospective cohort study ED / EED diagnosis should be made no later than 2 years of age. However, performing biopsy by upper gastrointestinal endoscopy of young children is invasive and maybe regarded as unethical in most circumstances, implying the need for simple, non-invasive and low-cost method should be developed for the diagnosis of ED / EED in developing countries(83, 84). Urinary lactulose to mannitol (L: M) ratio is the most commonly used non-invasive method for the diagnosis of EE / EED in previous studies In one study, it was shown that L: M ratio is well correlated with histopathological change (intraepithelial cell lymphocyte number) .However, L: M ratio is not standardized among studies(80). It is also technically complicated for children, because it involves fasting before testing and urine collection for up to five hours. Shorter period of urine collection may cause in adequate sampling due to involuntary infant maturation(76, 85).

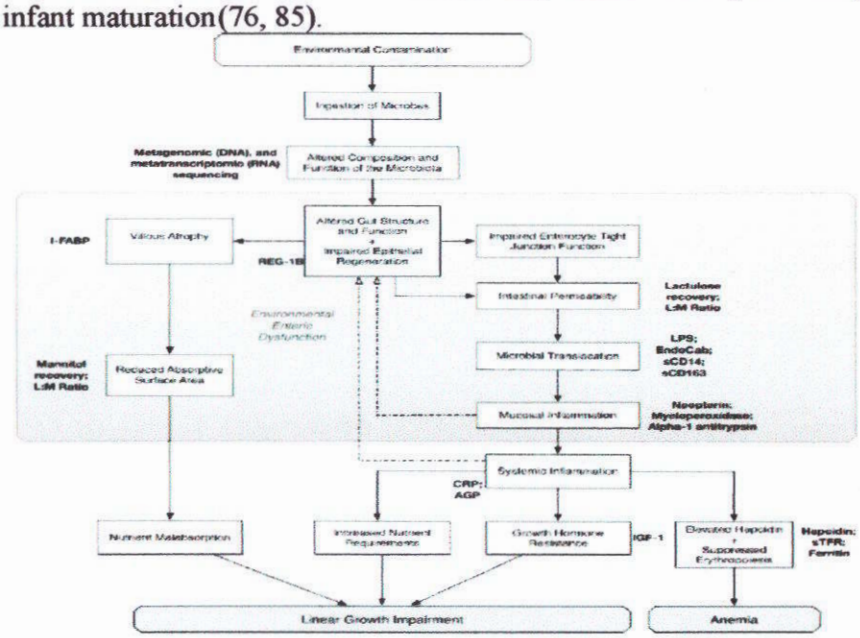


Figure 2. Hypothesized causal pathway to stunting through environmental enteric dysfunction. Abbreviations: AGP, α-1 acid glycoprotein; CRP, C-reactive protein; I-FABP, intestinal fatty acid binding protein; IGF-1, insulin-like growth factor 1; L:M, lactulose mannitol ratio; LPS, lipopolysaccharide; REG-1B, regenerating gene 1B; sCD14, soluble CD14; sCD163, soluble CD163; sTFR, soluble transferrin receptor.

Pathogenesis of EED:

The predicted EE / EED pathogenesis is illustrated in Figure 2. People living in poor sa nitary conditions are continuously exposed to intestinal bacteria, viruses and parasites by ingestion of food and water contaminated with feces (7). Ingested bacteria can stimulate the small intestine, leading to chronic inflammation of the gut accompanied by morphological chang es such as flattened villi and crypt hype Induces translocation of the bacteria followed by systemic inflammation and metabolic changes. Malabsorption, which may be explained by flattened villi, is often accompanied by changes in morphology of the small in testine leading to malnutrition (45, 84). It is assumed that these abnormalities are overlapping but not equitably occurring in each case. We have to realize which abnormality is present in each individual in order to design effective interventions.

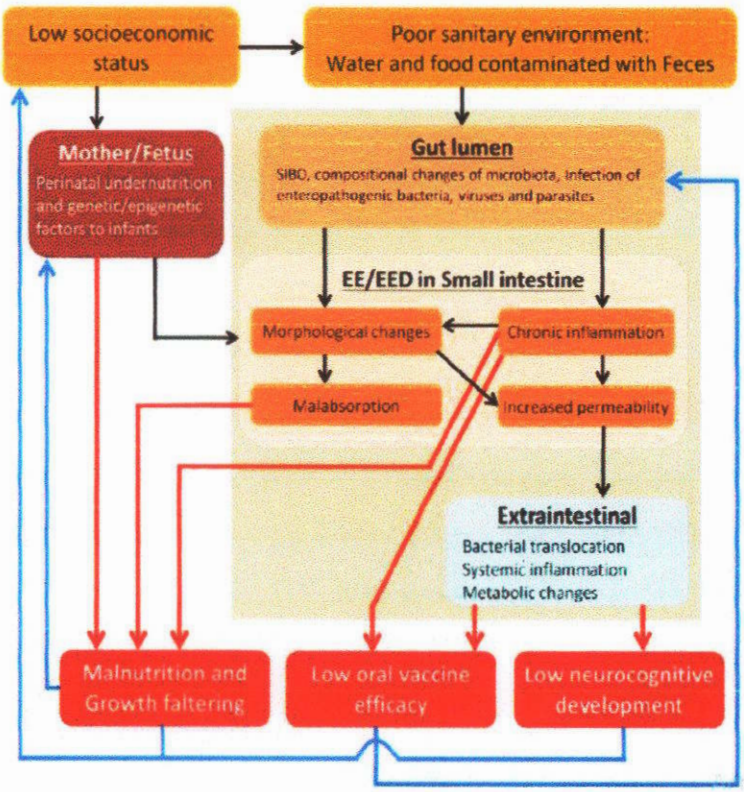


Figure 3. Pathogenesis of EE/EED and long-lasting consequences. Continuous exposure to fecal matter triggers EE/EED in individuals living in poor sanitary condition. Pathogenic conditions are seen systemically as well as locally in small intestine (black arrows). Pathogenic conditions have adverse effects on children's health as inducing long-lasting consequences (red arrows). These consequences, in turn, contribute a vicious cycle (blue arrows). Abbreviations,

EE, environmental enteropathy; EED, environmental enteric dysfunction; SIBO, small intestine bacterial overgrowth.

Impact of continuous exposure to fecal matter on gut microbiome in the small intestine:

Although continuous bacterial exposure from upper gastrointestinal tract to feces is likely to be the main cause of EE / EED based on epidemiological studies, it is still unclear how these enteropathogens trigger EE / EED growth (7, 86). Small intestine bacterial overgrowth (SIBO) is a subclinical quantitative abnormality of bacteria in the upper gastrointestinal tract defined by greater than 105 CFU / mL upper intestinal aspirate as assessed by both anaerobic and aerobic cultures (adhesions after surgery or radiation), inflammation (inflammatory bowel diseases and HIV infection), and metabolic disorders (diabetes mellitus), it is frequently seen in people living in developing countries (87).Importantly, it was also shown that SIBO is associated with growth faltering. An earlier study in Myanmar performing breath hydrogen test after rice meal as a diagnosis

Although SIBO is observed among patients with anatomic abnormalities showed 20.7% of the participants had SIBO and children having SIBO had a high relative risk of having faltered growth performed glucose hydrogen breath testing for 90 Bangladesh 2-year-old children, and showed a strong association between the presence of SIBO and higher intestinal inflammation markers (fecal REG1beta and fecal calprotectin). They also showed the presence of SIBO is negatively correlated with growth rate from birth to 2 year of age (delta length for age Z score from birth to 2 years of age)(56).

Chronic Immune activation in the gut :

Chronic exposure to polluted feces in food, water, and household environments causes an asymptomatic inflammatory response. There is one study in Gambia which conducted extensive histopathological analyzes(56). In this study, histopathological examinations of duodenal biopsy specimens including immunohistochemical staining were applied to 38 rural Gambian children (0.5–3 years)(56, 88). All children in Gambian had the EE / EED findings through histopathology. Though morphological changes were independent of nutritional status, the number of T cells increased and the number of B cells in the gut fell with nutrition worsening. They have also shown that the ratio of regulatory cytokines (TGF-βplusIL-10) over inflammatory cytokines (IFN-γ plus TNF-α) was decreased in malnourished children compared to well-nourished(88, 89).

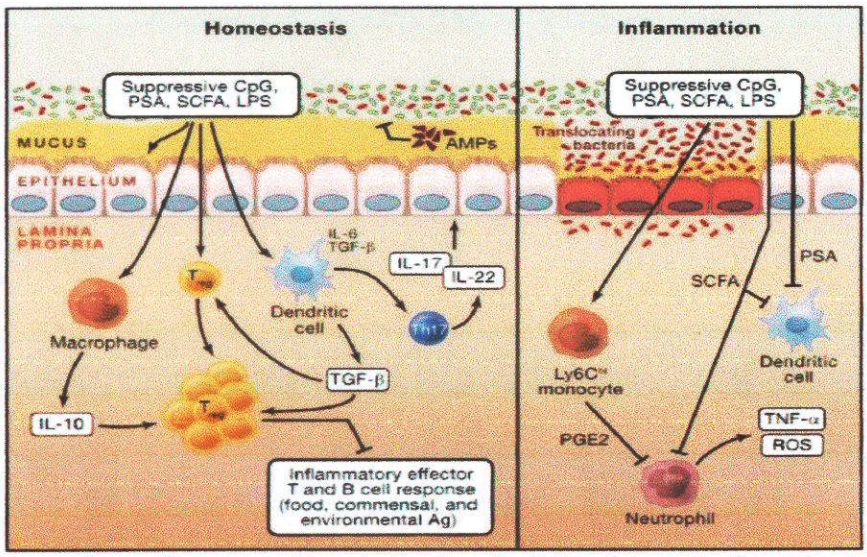


Figure 4: Promotion of Immune Regulation by the Microbiota during Steady State and Inflammation (Left) Commensals promote the induction of regulatory T cells via direct sensing of microbial products or metabolites by T cells or dendritic cells. Further commensals promote the induction of Th17 cells that can regulate the function and homeostasis of epithelial cells. In the context of inflammation, similar mechanisms may account for the regulatory role of the microbiota. (Right) Commensal-derived metabolites can also have a local and systemic effect on inflammatory cells. For example, SCFA can inhibit neutrop hil activation. Upon entrance in the tissue, inflammatory monocytes can also respond to microbial-derived ligands by producing mediators such as PGE_2 that limit neutrophil activation and tissue damage.

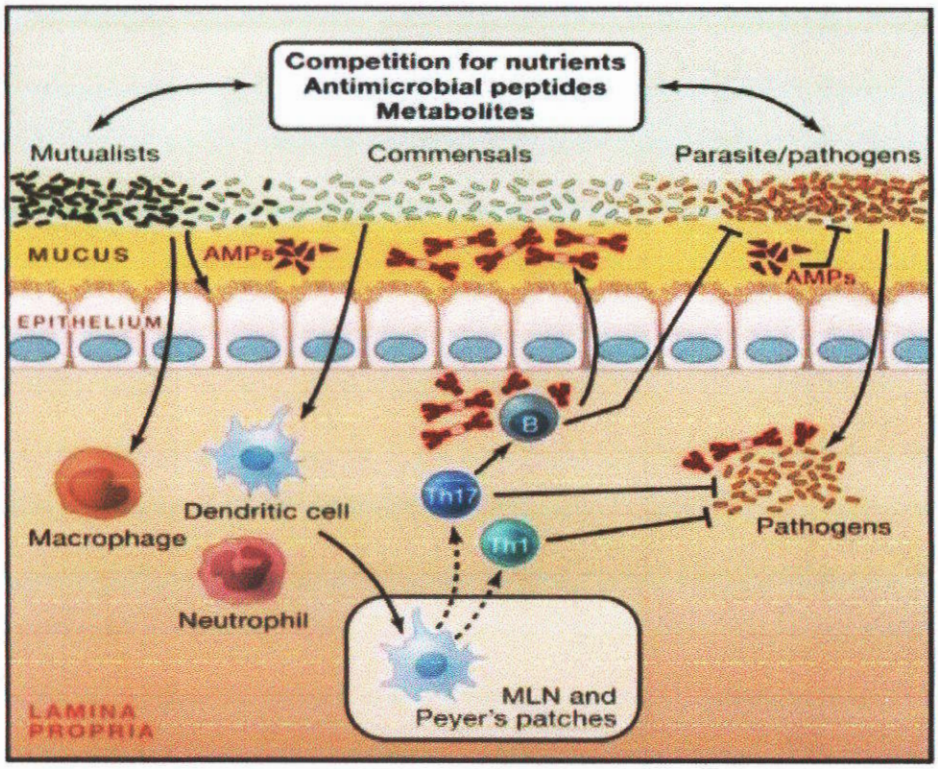


Figure 5: Promotion of Protective Immunity by the Microbiota: The symbiosis between the microbiota and its mammalian host encompasses multiple relationships, including mutualistic, parasitic, and commensal. The capacity of a given microbe, including those composing the microbiota, to trigger or promote disease is highly contextual, and some microbes can shift from mutualist to commensal to parasite according to the state of activation of the host, co-infection, or localization. Commensals can control microbes with pathogenic potential (as normal constituent of the microbiota or acquired) via distinct mechanisms. Commensals can compete for nutrients and produce antimicrobial molecules and metabolites that affect the survival and virulence of pathogens. Commensals can promote the production of antimicrobial peptides by epithelial cells and reinforce tight junctions. Finally, commensals can modulate the function of dendritic cells and other innate cells both locally and systemically in a manner that promotes the induction of effector T and B cells responses against pathogens. When uncontrolled, this adjuvant property of the microbiota can promote inflammatory and autoimmune disorders.

Bacterial translocation:

High intestinal permeability causes the translocation of bacteria into the blood. In the study of rural Gambian infants aged between 8 and 64 weeks, endotoxin plasma concentrations were elevated and contributed to both growth faltering and histopathology measures of mucosal enteropathy(88). This study also demonstrated that immunoglobulin-G-endotoxin core antibody was negatively correlated with height growth (cm / month) and positively correlated with gut permeability (L: M ratio). These results indicate that increased gut permeability induces translocation of bacterial components into blood and induces immune (56).

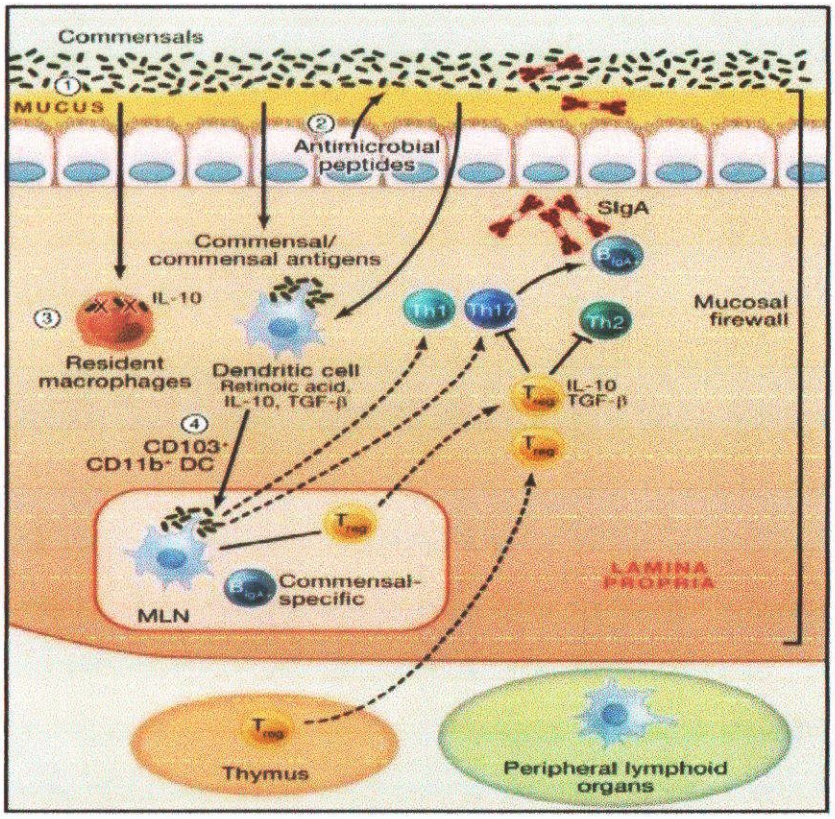


Figure 6: The mucus represents the primary barrier limiting contact between the microbiota and host tissue preventing microbial translocation. (2) Epithelial cells produce antimicrobial peptides that also play a significant role in limiting exposure to the commensal microbiota. (3) Translocating commensals are rapidly eliminated by tissue-resident macrophages. (4) Commensals or commensal antigens can also be captured by $CD103^+$ $CD11b^+$ DCs that traffic to the mLN from the lamina propria but do not penetrate further. Presentation of commensal antigens by these DCs leads to the differentiation of commensal-specific regulatory cells (T_{reg}), Th_{17} cells, and IgA-producing B cells. Commensal-specific lymphocytes traffic to the lamina propria and Peyer's patches. In the Peyer's patches, T_{reg} can further promote class switching and IgA generation against commensals. The combination of the epithelial barrier, mucus layer, IgA, and DCs and T cells comprises the "mucosal firewall," which limits the passage and exposure of commensals to the gut-associated lymphoid tissue, preventing untoward activation and pathology.

Consequences of EED

Malnutrition and growth faltering:

Although strategies of breastfeeding promotion for mother and providing sufficient food for children have a large effect on survival, their effect on stunting is small .For all developing countries, it is estimated that 32% and 20% of children (b5 years old) are under-height (height for-age Z score) and under-weight (weight-for-age Z score) for age (56). In addition, it was shown in the 1991 Gambian cohort that the persistent increase in intestinal permeability between 2 and 15 months of age was correlated with a falteri ng growth, regardless of diarrheal

episodes .These results help us to arrive at the awareness that not only sufficient food and adequate breast feeding but also prevention for chronic gut inflammation by EE / EED are essential to achieve expected growth in children living in developing world. Furthermore, linear growth faltering occurs within the first two years of life, and it is irreversible(88). Considered together, it is necessary to diagnose EE / EED as early as possible in their life.

Besides, The effects of EED on growth are difficult to isolate, as many of the hypothesized causes of the condition are also linked directly to impaired growth, but they are important to consider nonetheless, as the crux of the EED hypothesis is that the effects of growth -limiting exposures, such as inadequate diet and repeated infectious morbidities, are underestimated when the full burden and consequences of EED are not included. Studies in children admitted to treatment programs for acute malnutrition have generally found high rates of impaired gut integrity on admission and improvements in gut integrity markers over the course of recovery (90,111,121). Non-hospital-based prospective studies of gut integrity and linear growth have been fewer and those that have been published report inconsistent findings(93,109) .Still, evidence supporting a relationship with growth has been accumulating in recent years. One preliminary report from the MAL-ED multi-country cohort provides longitudinal data attributing poorer six-month growth in children age three to15 months to a score of fecal intestinal inflammation and permeability markers(20). A study from urban Bangladesh found increased risk of stunting at age 12 months with each unit increase in EndoCAb measured at age six months,(20) and another in Malawi found an inverse association between urinary lactulose recovery and rate of height-for-age z-score change in slightly older children over a three month follow-up period.(65) Additionally a post-hoc case-control analysis of blood samples from stunted and non-stunted HIV-negative 18-month-old children in Zimbabwe found evidence of persistent inflammation from six weeks to 12 months of age, confirming that inflammation in otherwise healthy children can be chronic in this setting and is associated with stunting.

Vaccine failure (Changes in gut immune responses and systemic inflammation):

It has been shown that many oral vaccines, both live and non-living, are less immunogenic and less protective when administered to individuals in developing countries than those in developed countries(90). The PROVIDE study used several makers of systemic inflammation and gut inflammation early in life, and tested the relation between these markers and vaccine efficacy. Gut inflammation and systemic inflammation were closely related to oral vaccine underperformance whereas systemic inflammation was positively associated with better parenteral vaccine responses. This finding underlined that low oral vaccine efficacy in developing countries is due, at least in part, to chronic gut and systemic inflammation. On the other hand, recent clinical trials of oral polio vaccine effectiveness after antibiotic treatment could not show better OPV efficacy although the antibiotics improved inflammation of the gut(91).

Neurocognitive development failure:

It was shown that enteric parasite infections, such as Giardia and Cryptosporidium and diarrheal episodes as well as stunting during early life are associated with poor neurocognitive development in later life(92) .Also animal models have shown that a perinatal in Systemic inflammation induced by intravenous administration of lipopolysaccharide (LPS) in early life

(14 days after birth) altered emotional behaviour at adolescence (30 days after birth) and adulthood (90 days after birth) in the murine model(92, 93). In this study, decreased phosphorylation of the glucocorticoid receptor in the prefrontal cortex of brain was shown in mice treated with LPS. These studies suggest that systemic inflammation results in subsequent poor neurocognitive development.

Gut Barrier Function:

A fundamental function of the intestinal epithelium is to act as a barrier that regulates interactions between the luminal contents, such as the intestinal microbiota, the underlying immune system, and the remainder of the body, while supporting vectorial transport of nutrients, water, and waste products. Epithelial barrier function requires a contiguous layer of cells as well as the junctions that seal the paracellular space between epithelial cells. Compromised intestinal barrier function has been associated with a number of disease states, both intestinal and systemic.

The intestinal tract contains the body's largest interface between a person and his or her external environment. The complexity of its function is obvious when thinking that at the same time the intestine must serve two opposite functions; the selective permeability of needed nutrients from the intestinal lumen into the circulation and into the internal milieu in general and, on the other hand, the prevention of the penetration of harmful entities including microorganisms, luminal antigens, and luminal proinflammatory factors. The latter function is known as barrier function (9).

The gut barrier function is comprised by three major lines of defence (36) The biological barrier, which is made up of normal intestinal flora (gut microbiota) responsible for colonization resistance; 2) The immune barrier, which is composed of gut associated lymphoid tissue (GALT), effector and regulatory T cells, IgA producing B (plasma) cells, group 3 innate lymphoid cells, and, resident macrophages and dendritic cells in the lamina propria; and 3) The mechanical barrier, consisting of the closed-lining intestinal epithelial cells and by the capillary endothelial cells. The epithelial and endothelial cells come into the closest possible contact in the most apical part of the lateral cell membranes ("kissing points") by specific structures named "tight junctions" (TJs), which interconnect the cells and restrict the passage of ions, molecules and cells through the paracellular space (45,76).

The term "bacterial translocation" (BT), was first described by Berg and Garlington in 1979, as the phenomenon of passage of viable bacteria from the gastrointestinal tract through the epithelial mucosa into the lamina propria and then to the mesenteric lymph nodes and possibly other normally sterile organs (37). This initial definition was later widened to include the translocation of non-viable bacteria or their products, namely pathogen-associated molecular patterns (PAMPs), with main representative the intestinal endotoxin. BT occurs in healthy individuals in a low rate of 5-10%, serving two main physiological roles; the antigenic exposure of the gut immune system to be prepared for an effective immune response in case of extensive pathogen invasion, and the development of immune tolerance to several microbial antigens of commensal microflora (36).

The intestinal barrier is compromised in several disease states leading to an increased level of BT associated with infectious complications and promotion of a systemic inflammatory response that aggravates the pathophysiological consequences of the underlying disease (23,45). There are three main pathophysiological groups of intestinal barrier failure associated with pathologic conditions:

1) The intestinal barrier failure observed in surgical patients subjected to major operations for diverse reasons (major liver resections, bowel resections for malignancy, bowel transplantation, aortic aneurysm repair). In this group of patients, increased BT is associated with increased postoperative infectious complications (38). The connecting mechanism is translocation of gut-derived pathogens through a dysfunctional mucosal barrier to the mesenteric lymph nodes, the portal vein and the systemic circulation, eventually leading to postoperative infections. Also, this is the mechanism by which the necrotic p ancreas becomes infected in patients with severe necrotic pancreatitis (32).
2) The second group includes critically ill patients, severely injured or septic, hospitalized in intensive care units. Increased gut permeability is associated with the developme nt of systemic inflammatory response and multiple organ dysfunction syndrome (MODS) in these patients. However, the connecting pathophysiological link of gut barrier failure and MODS does not seem to be the classical process of BT (62). Current pathogeneti c aspects support the "gut-lymph" theory of sepsis and MODS. According to this theory, microbes and/or their products, through a dysfunctional gut barrier, first gain access to the intestinal submucosa activating the intestinal immunological system of defence. An intestinal proinflammatory response further aggravates intestinal injury and danger-associated molecular patterns (DAMPs) are released in the mesenteric lymphatics, carried to the lung and the systemic circulation, stimulating Toll like receptors-4 and perhaps other pattern recognition receptors (PRR) in a fashion similar to bacteria, thus eventually promoting injurious effects in diverse organs. Therefore, the gut becomes a pivotal proinflammatory organ promoting deleterious effects in even distant organs, through release of DAMPs, without the need of systemic bacterial translocation (28).
3) The third group of intestinal barrier dysfunction involves stable patients with chronic pathologic conditions that present a low-grade translocation of enteric microbes and immunostimulatory bioproducts from the gut lumen first in the lamina propria and thereafter in the systemic circulation, promoting a chronic immune activation associated with disease progression and/or development of complications and comorbi dities from other organs . This intestinal barrier dysfunction group encompasses patients with HIV infection, liver cirrhosis, chronic viral hepatitis B or C, non-alcoholic steatohepatitis or non-alcoholic fatty liver disease, patients with inflammatory bowel diseases, celiac disease, irritable bowel syndrome, obesity and diverse autoimmune conditions. For example, in HIV infection, intestinal barrier dysfunction, BT and chronic immune activation have been associated with cardiovascular, neurocognitive and lymphoproliferative comorbidities, despite effective viral suppression with modern antiretroviral treatment; and in liver cirrhosis intestinal barrier dysfunction has been associated with all of its complications, namely spontaneous bacterial peritonitis, hepatic encephalopathy, hepatorenal syndrome, hepatopulmonary syndrome, variceal bleeding, progression of liver injury and hepatocellular carcinoma (34,38).

Interface or Barrier:

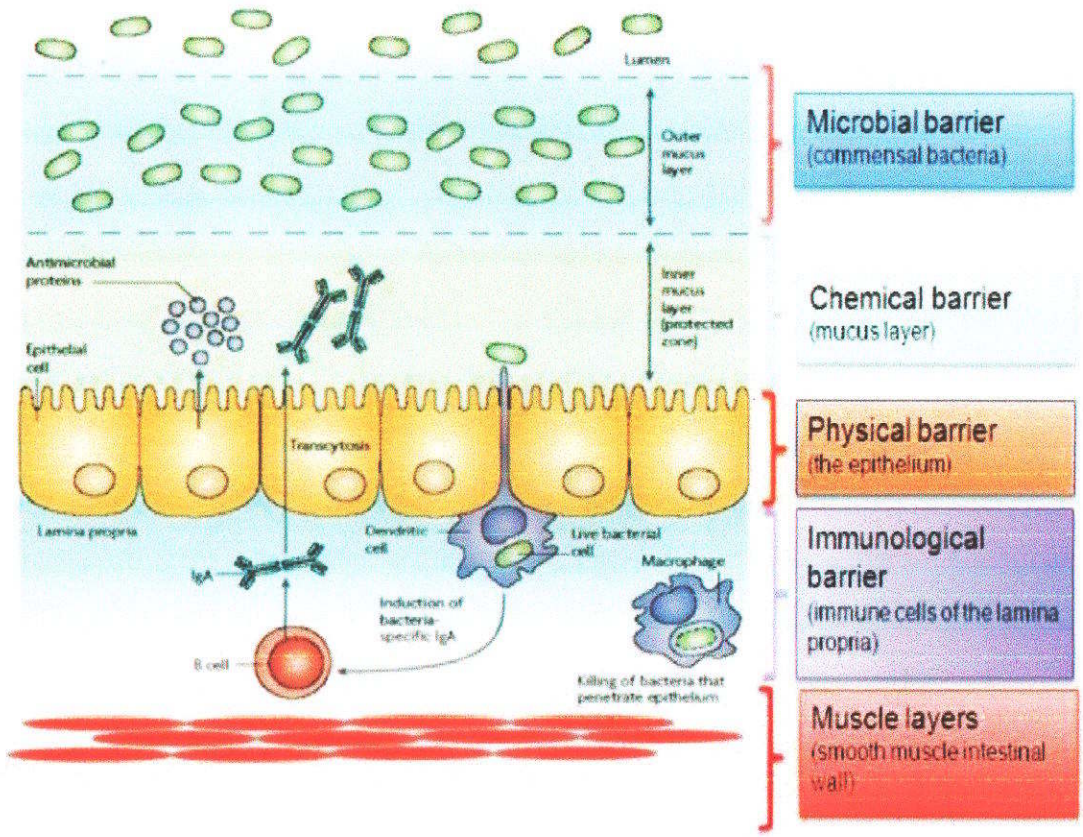


Figure 7: The lower gastrointestinal tract consists of the small and large intestines which are referred to as the gut. The gut has a multi-layer system made up of gut microbiota, mucus, and epithelial cells. The microbial barrier or the gut flora consists of comme nsal friendly or probiotic bacteria that not only provide nutrients such as ferment fiber, release short -chain fatty acids, and synthesize other key nutritional components such as Vitamin K2, it also suppresses the growth of unfriendly bacteria such as yeast. The mucus layer is produced by the Goblet cells in the intestinal lining which prevent bacteria from adhering to the gut lining and regulates diffusion to the host tissues by filtering the toxins from being absorbed. The intestinal epithelial layer forms the most important barrier between the internal and external environment. The tight junctions are responsible for sealing the intercellular space and functions as a selective/semipermeable barrier that facilitates the passage of ions and solutes while s topping the passage of antigens and toxins. The immunological barrier sorts between pathogens and bacterial microorganisms as well as regulates the immune tolerance and response to pathogens.

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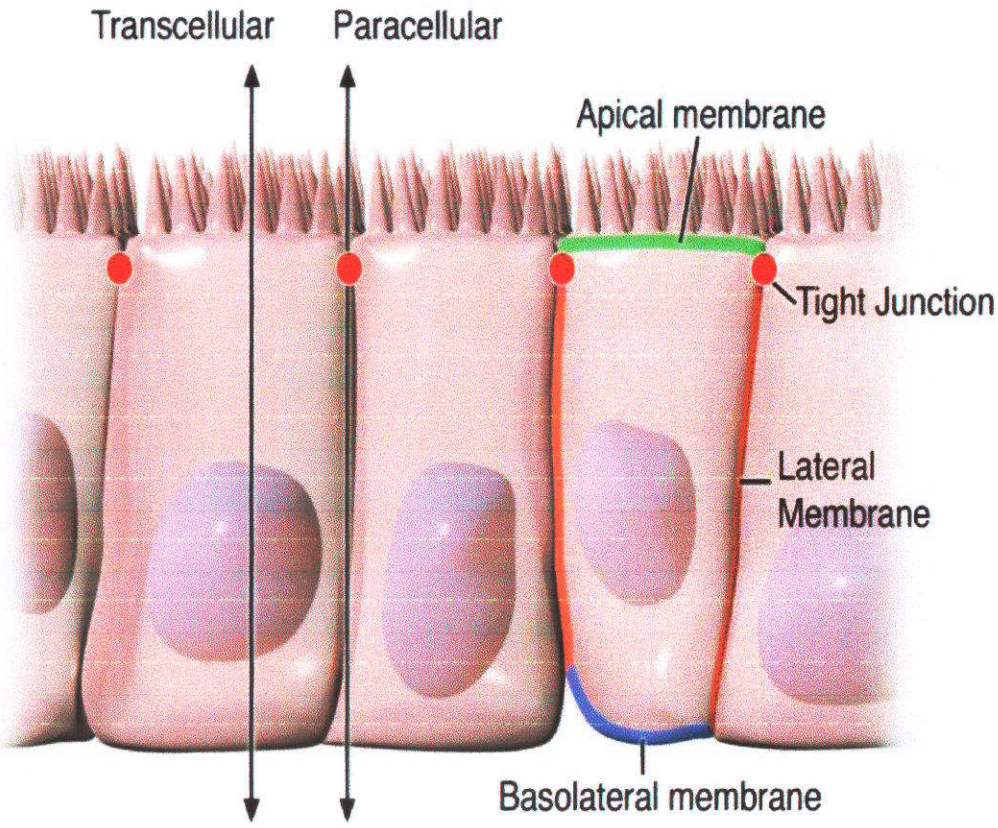


Figure 8. Pathways of epithelial permeability

Transcellular permeability is associated with solute or water movement through intestinal epithelial cells. Paracellular permeability is associated with movement in the intercellular space between epithelial cells and is regulated by tight junctions localized at the junction of the apical-lateral membranes(94).

The intestinal epithelium consists of a single layer of polarized epithelial cells. Adjacent cells are connected by 3 main junctional complexes: desmosomes, adherens junctions and tight junctions(45). Desmosomes are localized dense plaques that are connected to keratin filaments. Adherens and tight junctions both consist of transcellular proteins connected intracellularly via adaptor proteins to the actin cytoskeleton. The collection of proteins in the junctiona l complexes form "cytoplasmic plaques".

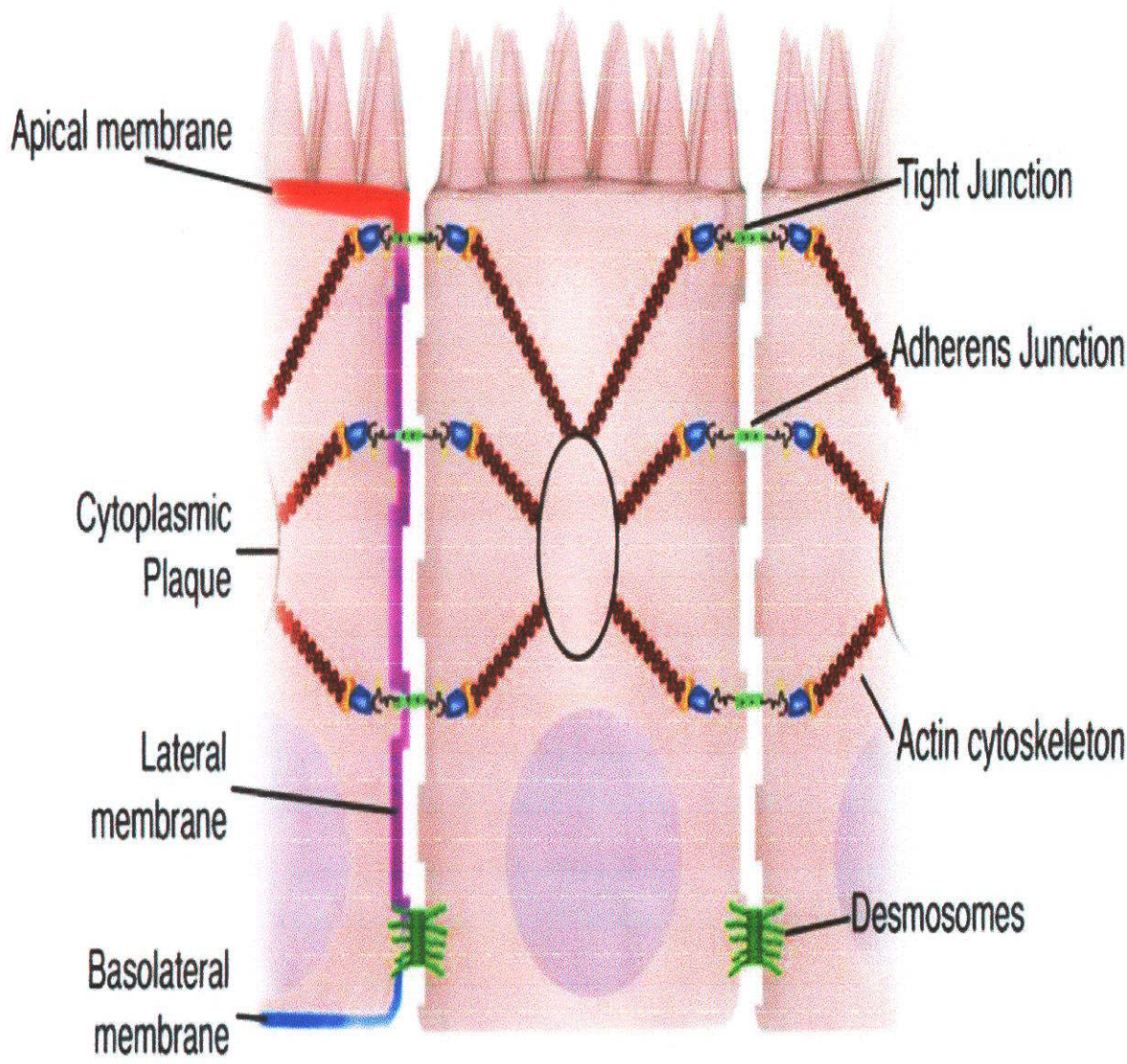


Figure 9. Overview of intestinal epithelial junctional complexes

The intestinal epithelium consists of a single layer of polarized epithelial cells. Adjacent cells are connected by 3 main junctional complexes: desmosomes, adherens junctions and tight junctions. Desmosomes are localized dense plaques that are connected to keratin filaments. Adherens and tight junctions both consist of transcellular proteins connected intracellularly via adaptor proteins to the actin cytoskeleton. The collection of proteins in the junctional complexes form "cytoplasmic plaques".

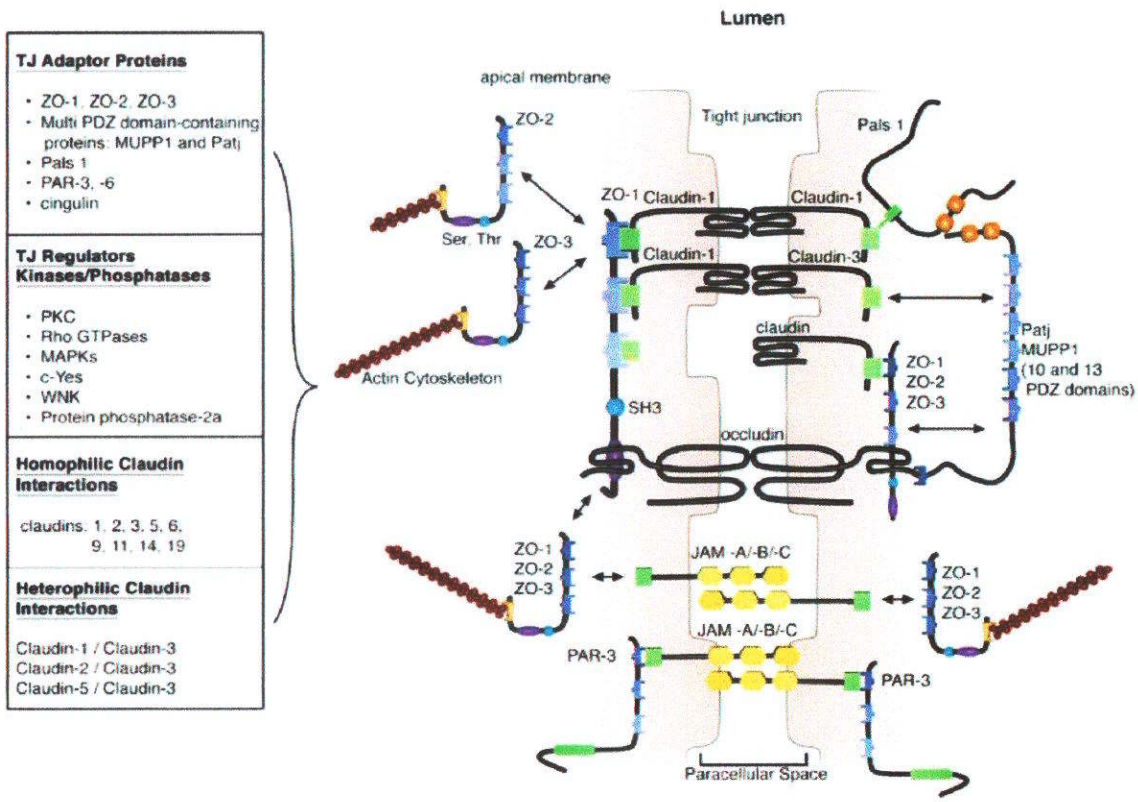


Figure 10. Tight Junctions

Tight junctions (TJs) are localized to the apical-lateral membrane junction. They consist of integral transmembrane proteins (occludin, claudins and junctional adhesion molecules (JAMs) that interact in the paracellular space with proteins on adjacent cells. Interactions can be homophilic (e.g. claudin-1/claudin-1) or heterophilic (claudin-1/claudin-3)(95). The intracellular domains of transmembrane proteins interact with PDZ-domain-containing adaptor proteins that mechanically link the TJ complex to the actin cytoskeleton. TJ proteins are regulated by phosphorylation by kinases, phosphatases and other signaling molecules(7).

Assessment of gut barrier function:

Lactulose Mannitol Test

The lactulose mannitol test is the current gold standard of testing for leaky gut. It is non-invasive, the results are accurate and easy to interpret, and it's relatively inexpensive to carry out. The lactulose: mannitol (LM) test is a quantitative assay for directly measuring the ability of two non-metabolized sugar molecules—lactulose and mannitol—to permeate the intestinal mucosa. Mannitol, a readily-absorbed monomer, serves as a marker of trans-cellular uptake; and lactulose, a dimer, is only slightly-absorbed and serves as a marker for mucosal integrity. An elevated lactulose to mannitol ratio is an indicator of intestinal barrier dysfunction(96). The LM test can be used as a non-invasive diagnostic aid to detect intestinal barrier dysfunction in patients with diarrheal diseases malnutrition , HIV infection , trauma , surgical stress , celiac disease , Crohn's disease , active inflammatory bowel disease (IBD), irritable bowel syndrome

(IBS), allergy and intolerance to cow's milk , lactase deficiency, mucositis induced by chemotherapy or inflammatory agents , as well as a sensitive parameter to follow nutrition or sanitation related intervention studies to assess gastrointestinal health of children living in poverty(97).

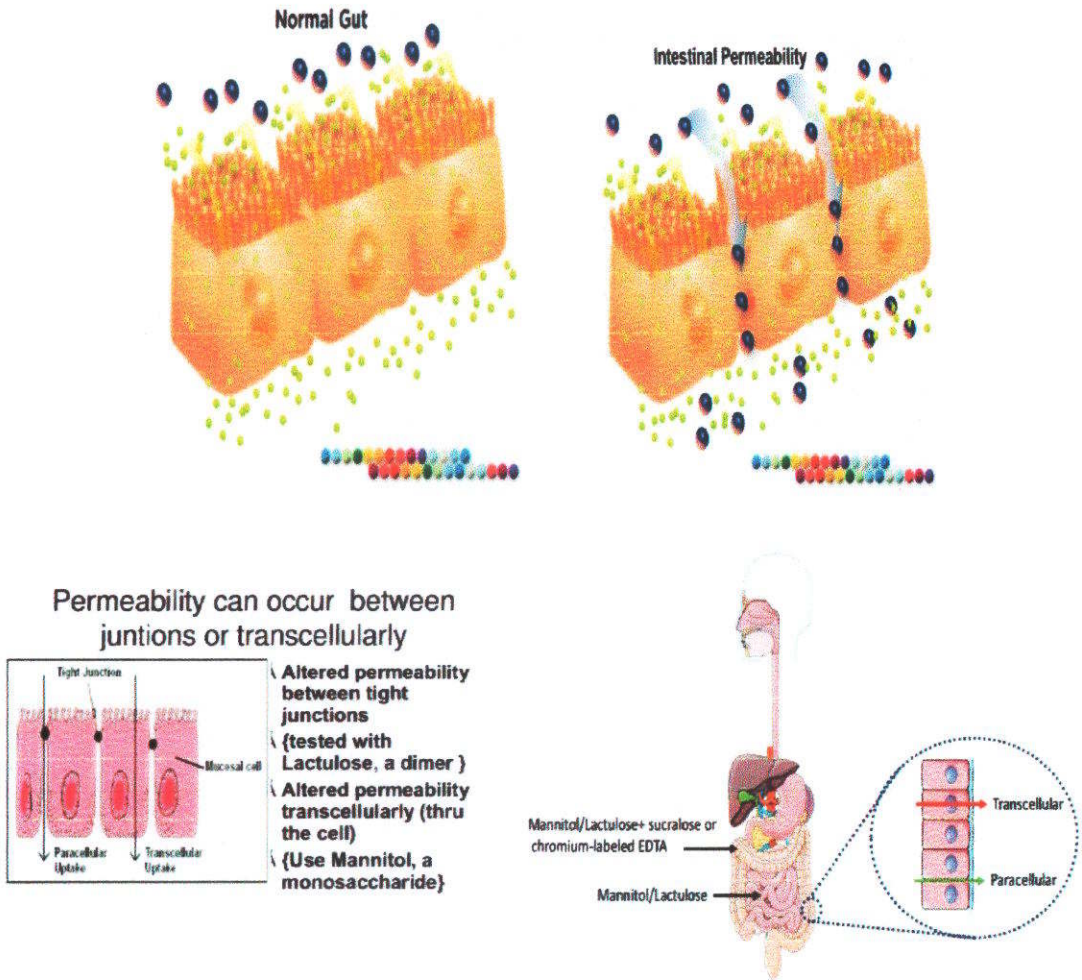


Figure 11: Evaluation of intestinal permeability. Urinary excretion of orally ingested non-metabolizable sugars of different sizes provides a reliable non-invasive *in vivo* read-out of intestinal barrier function. The mannitol/lactulose ratio evaluates the changes in permeability in the small intestine. Changes in the colon permeability is assessed with the addition of either sucralose or chromium-labeled EDTA. At the cellular level, there are two routes for transport of molecules and ions across the epithelium of the gut: across the plasma membrane of the epithelial cells (transcellular route) and across tight junctions between epithelial cells The gastrointestinal tract is not only the primary organ of digestion and absorption but also the largest area of exposure in the human body to the external environment. The feature known as gut barrier has to do with the integrity of the gastrointestinal tract and, at t he same time, with the relationship between the intestinal lumen microbiota and the host. The concept of gut barrier is associated with permeability and is related to the property of a membrane that enables

the passage of a solute by unmediated diffusion. On the other hand, absorption describes carrier-mediated modes of transportation of substances such as sodium and chloride across the intestine. After the introduction of non-metabolizable oligosaccharides as test substances in the 1970s, the development of methods for assessing intestinal barrier function became feasible. The oral administration of sugars and the subsequent assessment of these compounds in the urine, denoted as the intestinal permeability test, is a non-invasive procedure used to determine epithelial barrier integrity.

C-reactive protein (CRP)

C-reactive protein is a homo-pentameric acute-phase inflammatory protein, a highly conserved plasma protein that was initially discovered in 1930 by Tillet and Francis while investigati ng the sera of patients suffering from the acute stage of Pneumococcus infection. In the presence of calcium, CRP binds to polysaccharides such as phosphocholine (PCh) (96)on microorganisms and triggers the classical complement pathway of innate immunity by activating C1q CRP has many homologs in vertebrates and some invertebrates and is a member of the pentraxin family, which includes other structurally related molecules such as serum amyloid A .The average levels of CRP in serum in a healthy Caucasian is around 0.8 mg/L, but this baseline can vary greatly in individuals due to other factors, including polymorphisms in the CRP gene (23). The human CRP gene can be found at 1q23.2 on the long arm of chromosome 1, and to date, there have been no allelic variations or genetic deficiencies discovered for this gene although some polymorphisms have been identified

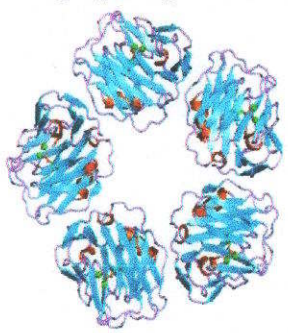


Figure 12: Three-dimensional structure of C-Reactive Protein

The majority of research on CRP has focused on the role of CRP and its isoforms on cardiovascular disease and stroke. CRP is used as a clinical marker of inflammation, with elevated serum levels being a strong independent predictor of cardiovascular disease in asymptomatic individuals(98-100). CRP levels have been linked to prognosis in patients with atherosclerotic disease, congestive heart failure, atrial fibrillation, myocarditis, aortic valve disease, and heart transplantation, suggesting that it has an active role in the pathophysiology of cardiovascular disease. High sensitivity assays, such as nephelometric assays, are used to detect baseline levels of CRP and patients who are at risk of cardiovascular disease. An individual with a CRP level higher than 3 mg/L has an increased risk of coronary heart disease (42), and this risk increases in those with type 2 diabetes. Increased levels of CRP have been found in patients with appendicitis, cholecystitis, pancreatitis, and meningitis.

C-reactive protein is a marker for inflammation, and its levels increase during bacterial infection. Kingsley and Jones stated that CRP increases during infection in response to monocytic mediators such as IL-1 and IL-6 and that it has a stable decay rate. It is thought that

most of the interaction between CRP and the immune response to pathogens involves the binding of CRP to PCh and the activation of the classical complement pathway. showed that CRP provides mice with protection against infection by the gram -positive pathogen Streptococcus pneumoniae by binding to a PCh determinant of the pathogen cell wall and activating the complement pathway(15).

Role of C-Reactive Protein at Sites of Inflammation and Infection

CRP IN DISEASE PATHOLOGY

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CRP AND INFECTION

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CRP AND INFLAMMATION

Systemic inflammation may contribute to poorer growth in children and has been observed in connection with EED. Additionally, repeated symptomatic illness, especially diarrhea, is one hypothesized casual factor in the development of EED. The serum proteins C -reactive protein (CRP) and α-1 acid glycoprotein (AGP) are elevated during the acute phase response, indicating a current systemic immune response.

Soluble CD14:

CD14 was first characterized as a membrane cell surface differentiation marker of myeloid lineage cells. Membrane CD14 is a GPI anchored 55 kDa glycoprotein that is highly expressed on monocytes, macrophages and neutrophils with lower surface expression observed on a variety of other hematopoietic and stromal cells. Soluble forms of CD14 exist in serum, cerebrospinal(101), and other body fluids. Soluble CD14 is generated by at least three different

mechanisms which include bypassing of GPI addition, cleavage of the GPI anchor by phospholipase D, or direct proteolytic cleavage from the cell surface (reviewed in CD14 is best known as a pattern recognition receptor of the innate immune system that plays a prominent role in sensitizing cells to the presence of Gram-negative bacterial lipopolysaccharide(26).

CD14 is thought to sensitize cells to LPS by delivering this agonist to the Toll -like receptor 4 (TLR4) receptor signaling complex. Membrane CD14 shuttles LPS to TLR4 complexes in a two-dimensional formation, whereas, soluble CD14 enables cells that lack endogenous CD14, including most epithelial and endothelial cells, to respond to LPS. LPS -induced TLR4 activation initiates host inflammation by driving cellular production of pro-inflammatory cytokines, chemokines and cell adhesion molecules. At the local site of infection, pro-inflammatory signaling resulting from CD14 shuttling is protective, leading to local clearance of invading bacteria. However, widespread infection and activation of this pro -inflammatory signaling system can cause fatality through sepsis. In response to LPS, CD14 deficient mice have blunted pro-inflammatory cytokine production and lack the lethargy, re spiratory, and ruffled fur symptoms associated with murine septic shock. CD14 deficient mice also survive challenge with otherwise lethal doses of LPS. Lipopolysaccharide is the major lipid present in the outer membrane of Gram-negative bacteria(96). CD14 is a ubiquitous pattern recognition receptor. As the cell membrane receptor of bacterial LPS, CD14 plays a key role in a series of pathological reactions caused by immune and defence systems(88, 102).

The Function of CD14:

CD14 is a ubiquitous pattern recognition receptor. As the cell membrane receptor of bacterial LPS, CD14 plays a key role in a series of pathological reactions caused by immune and defense systems.

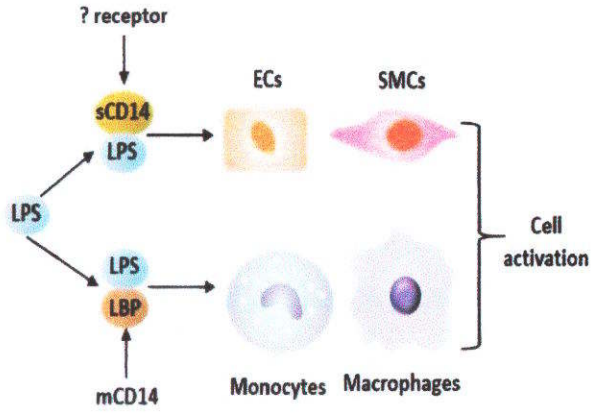


Figure 13: LPS interacts with LBP and CD14 to induce cell activation

Preventing environmental enteric dysfunction

Based on the observational and mechanistic evidence available, poor sanitation and hygiene contribute to, and are possibly the overriding cause of, EED Plausibly, avoiding the ingestion of enteric pathogens and any other causative microbes by infants and young children could prevent most of the EED burden.

What microbes are important in the aetiology of EED, i.e. commensal vs. pathogenic microbes This question is important in defining the range of environmental microbes and microbial products that interventions should address. Among 40 asymptomatic infants with EED living in an urban Sau Paulo slum, 63% had colonic bacterial proliferation of the small bowel Moreover, because only pathogenic bacteria cause clinical diarrhoea, sanitation/hygiene messages have often particularly focused on avoiding exposure to faeces of young children – the major carriers of these organisms However, it may be that high concentrations of *any* bacteria in the small bowel can cause EED: in experiments comparing germ-free with conventional animals, commensal bacteria in *low* concentrations exert a trophic effect on the intestinal epithelium shifting morphology from 'supernormal' (i.e. very tall villi) to normal, which results in a structurally and functionally competent immune system .Thus, high exposure to all faeces (including those from healthy people and animals) may contribute to EED. Chronic exposure to a contaminated environment creates a constant state of survival responses characterized by loss, malabsorption, maldigestion and inefficient utilization of nutrients. In the context of marginal diets and recurrent infections, this 'impoverished gut' condition likely explains a significant portion of the unresolved stunting affecting one in every three children in developing countries. To prevent stunting, we need to prevent the onset of EED because (i) EED is self-perpetuating once it has developed; (ii) recovery from EED is relatively slow even when there is a dramatic change in environment; and (iii) the window for critical growth and development is short (between conception and the first two years of postnatal life). Interventions such as baby-WASH, aimed at preventing and reducing environmental enteric dysfunction, may be central to global stunting reduction efforts.

The treatment of EED is fraught with difficulties. First, in the absence of robust point-of-care biomarkers, the identification of EED in the individual child is problematic. Moreover, there is no robust evidence from clinical trials that specific interventions can cure or ameliorate the signs and symptoms of EED. Because EED has its roots in the environment, the mainstay of preventing the condition is to "clean" the environment. This approach is challenging because water scarcity still afflicts 40% of the world's population, and 13%of the population still defecates in the open. 70 Furthermore, neither the world's people lack access to safe drinking water. The provision of basic sanitation facilities, potable water, and improved hygiene practices cut the chain of transmission of pathogenic bacteria that can colonize the small intestine and cause EED. Indeed, a study in Bangladesh found that ensuring clean environment increased population height-for-age 0.54 SD compared with children living in default conditions. 71 Moreover, stunting prevalence was reduced by 22%. More recently, a public sanitation program in Mali showed that enhanced access to toilets did not reduce the prevalence of diarrhoea increased childhood growth, particularly in those <2 years of age. 72 A possible explanation could be reduced chronic exposure to pathogenic bacteria, resulting in reduced severity or prevalence of EED. Ensuring hygiene at critical times is critical to preventing EED. A study conducted in slums and villages in Bangladesh revealed that 40% of complementary foods prepared by mothers were contaminated with *coli*; this contamination resulted in higher rates of diarrhoea and malnutrition. 73 It is now believed that zinc deficiency, rampant in developing countries, co-exists with EED and increases the severity of the condition. 20, 33, 34 Given the inadequate dietary intake of zinc in children living in developing countries, the importance of its supplementation, 74either long term or at least as part of treatment of diarrhoea, cannot be overestimated. The claim that exclusive breastfeeding can reduce gut inflammation was recently validated in South African children. 75In summary, emerging

evidence suggests that the following factors, in combination, can reduce the incidence, prevalence, and severity of EED: (1) access to safe drinking water and improved hygiene practices in low-income countries; (2) provision of sanitary toilet facilities and changes in public behaviour regarding their use; (3) exclusive breastfeeding for the first 6 months and continued breastfeeding thereafter; and (4) zinc supplementation. Because stunting is a hallmark of EED, a major goal should be to prevent the condition as well as treat its complications. In the context of poor socioeconomic conditions, prevent ive and therapeutic measures include increasing access of children to appropriate and adequate diets containing animal source foods, supplementation with zinc, and adequate treatment of recurrent illnesses such as diarrhoea pneumonia. Population wide prevention of EED will require adequate nutrition and health maintenance of all girls of reproductive age and women prior to, during, and after pregnancy. Under conditions of extreme food insecurity, supplementation with nutritious ready-to-use food for children with severe or moderate acute malnutrition has been shown to enhance clinical recovery. 76, 77Agents that can offset or reduce chronic inflammation at the gut mucosal level are currently being evaluated. These include5 - aminosalicyclic acid, which in a recent study was not efficacious 78; the nasal steroid budesonide; and an immunomodulatory small molecule called oglufanide disodium. 3Only well-designed, randomized controlled trials that assess efficacy and adverse effects in settings of high prevalence of stunting/EED can lead the way to effective and safe treatments.

Chapter Three: Materials and Methods

Period of study:

The period of study was from January 2019 to January 31, 2020.

Study area:

This study was carried out in slum area of Mirpur, Dhaka. This area was selected as the living condition is unhygienic and the impoverished children are mostly affected by diarrh eal disease than the others. All the laboratory works were done in the Parasitology Laboratory of icddr,b, Dhaka, Bangladesh.

Study population:

The population of the study was first 52 weeks children.

Sample size: Urine and Blood samples from 233 children of 52 weeks were collected for the study.

Materials and methods

Preparation of sugar stock solution:

a) weighed out accurately 0.10269g of lactulose, 0.10269 g melibiose and 0.05466 g mannitol and transfer into 100 ml volumetric flasks separately
b) added distilled de-ionized water up to 100 ml to make 3 mM sugar stock solution for each sugar
c) Kept @ 4C for short term or freeze @-20C for long term storage

Preparation of 5 levels of calibration standards (working standard) Working standard solution should prepared fresh daily.

Stock solution	Level-1	Level-2	Level-3	Level-4	Level-5	Final Concentration Level 1-5
Melibiose	50	50	50	50	50	50 µM for all levels
Lactulose	10	20	30	40	50	10, 20, 30, 40. 50 µM
Mannitol	10	20	30	40	50	10, 20, 30, 40. 50 µM
Distilled de-ionized water	to 3 ml	to 3 ml	to 3 ml	to 3 ml	to 3 ml	In 5 ml Tube

Preparation of samples

- Took out urine samples from the freezer and wait for thaw
- Vortexed the urine samples and centrifuge for 10 minutes @ 10000 rpm.
- After vortexing the samples was mixed , 100µl of supernatant urine and 50 µl of a solution containing melibiose (3.0 mM) diluted in 2.85 ml of distilled and Deionized water
- The final concentration of melibiose was 50µM.
- Vortexed both standard and urine samples
- Collected 700 µL of the above samples and standards to the Poly Vial with 20 µm Filter cap and placeed to the auto-sampler and read in the detector. Each sample can be automatically filtered during loading by an optional 20 µm filter in the vial cap, so that particulates can be removed during sampling.
- Both standards and urine samples automatically injected to the HPIC.
- Values were electronically transferred into the software program and results were calculated.

Preparation of Lactulose: Mannitol Solution for Ingestion by Study Subjects:

Objective

Prior to the administration of the LM test, prepare doses of lactulose and mannitol for ingestion by participating children. L:M solution contains lactulose (250 mg/ml) and mannitol (50 mg/ml) in a dose of 2 ml/Kg of weight or a maximum of 20 ml.

Materials

Balance
Mortar, Pestle
Petri dish
Spatula
Sterile capped tube/vial or a flask with metal top and stopper
Beakers - 100ml, 200ml, 2000ml
Magnetic stir bar, Magnetic stirrer
Pipetter, 1ml Pipette tips
Bunsen burner
70% ethanol
Sterile water
Mannitol (Sigma, Product of china, cat #M4124-1KG)
Lactulose solution USP 3.40 gm/5ml (680 mg/ml) (Lactulose syrup 200ml, each 5 ml concentrated oral Solution contains Lactulose USP 3.40 gm. Manufactured by: SQUARE Pharmaceuticals Ltd. Bangladesh. (www.squarepharma.com.bd)

Methodology

Pharmacist (or local equivalent) or pharmacist technician (under the direct supervision of the pharmacist) prepared and packaged the study agent in accordance with local pharmacy standards.

a) Preparation process:

1. Preparation of the lactulose/mannitol solution will be performed in the agent packaging room, entrance to which is limited to workers wearing appropriate clothing (clean lab coat, hat, mask, shoe covers, gloves). Work benches will be sterilized with 70% ethanol prior to preparation of study agents.
2. All supplies are autoclaved prior to use: stir bars, spatula, beakers, flasks, stoppers, gauze, water
3. Determine the volume of L:M solution to be prepared based on the L:M list,

Final concentration of 20 mL Lactulose: Mannitol = 1 g mannitol + 5 g lactulose

	500 ml	750 ml	1000 ml
Sterile water (mL)	316	474.27	632
Mannitol (g)	25	37.5	50
Lactulose solution (mL)	184	275.73	368

4. Added sterile water to a sterile beaker. Weigh mannitol and add to water. Mix for 15 minutes using magnetic stir bag.
5. After dissolving mannitol completely, measured lactulose solution and add it to the beaker. Mixed for an additional 15 minutes.
6. Aliquoted L:M solution into PID#-labeled sterile vials according to the weight of each subject (at a dose of 2 ml/Kg of weight or a maximum 20 ml). For example, a 6 kg infant would receive 12 ml of the L:M solution.

The sample randomly included 52 weeks children. Each participant or his / her guardians signed an informed consent form. The kids had always been healthy. It excluded from this study any children suffering from any diarrheal or fever diseases, congenital defects. Fasted children (at least 2 hours) were instructed to drink a solution containing lactulose (250 mg / ml, Osmolax, Square Pharmaceutical Ltd, Dhaka, Bangladesh) and mannitol (50 mg / ml, Sigma, St. Louis, MO, USA) in a dose of 2 ml / Kg of body weight, or a maximum 20 ml. The kids were asked to empty their bladders before they ingested the sugar test solution. To collect their urine, a pediatric urine collection bag (Fisher cat. #22275347, or Hollister U -bag #7501) was attached to the children. The children were allowed to return to their regular diet 30 minutes after ingestion of the LM test solution and 2 ml of urine was collected from the collection bag after 2 hours. Maximum urine volume was assessed 5 hours. 1–2 drops of thimerosal (Sigma

Aldrich) were added and samples processed until lactulose and mannitol kept in a -80 ° C freezer.



Figure14: Lactulose

HPIC (High Pressure Ion Chromatography): For HPIC testing, 100µL of each urine sample and 50µL of a solution containing melibiose (3.0 mM) diluted in 2.85 mL of distilled and deionized water. The final concentration of melibiose is 50 µM. The mobile phase should be filtered through a 0.45-µM millipore filter. All samples (500 µL each) were transferred to sample vial with optional 20 µm filter cap and placed to the auto sampler from where samples were automatically injected to the HPIC system. A 25 µL volume of each sample is injected automatically using the AS-DV sampler. Five levels of calibration standards were run with each batch to generate a standard curve using Chromeleon software. The Dionex ICS-5000 carbohydrate analyzer HPIC system (Dionex Co., Sunnyvale, CA, USA) is composed of the following modules: AS-DV Automated Sampler for injections, SP5000 Gradient Pump, DC Detector/Chromatography module with column and ED Electrochemical Detector with integrated Pulse Amperometry Detector. A Dionex CarboPac MA1 anion-exchange column (250 x 4.0 mm, Thermo scientific,BioLC) with an associated CarboPac MA1 guard column (50 x 4.0mm) is also from Dionex. Elution of the monosaccharides and disaccharides is achieved with an isocratic eluent of 450 mM NaOH at a flow-rate of 0.4 ml/min using the Dionex ICS5000 SP and MA1CarboPac column. Column temperature is set at 26 °C. Detection uses the ED5000 detector with a waveform Carbohydrate (standard Quad) consisting of the following potential-duration profile: sampling = 0.1 V, 200 ms; oxidation = 0.10 V, 400 ms; reduction = -2 V, 410 ms and -2 V, 420 ms; oxidation = 0.6 V, 430 ms; reduction = -0.1 V, 440 ms and -0.1 V, 500 ms. Output range of the detector is set at 1.0 mA with integration response time of 3 seconds as previously reported. Sugars were identified and measured using Chromeleon 6.8 software package (Dionex Co., Sunnyvale, CA, USA).

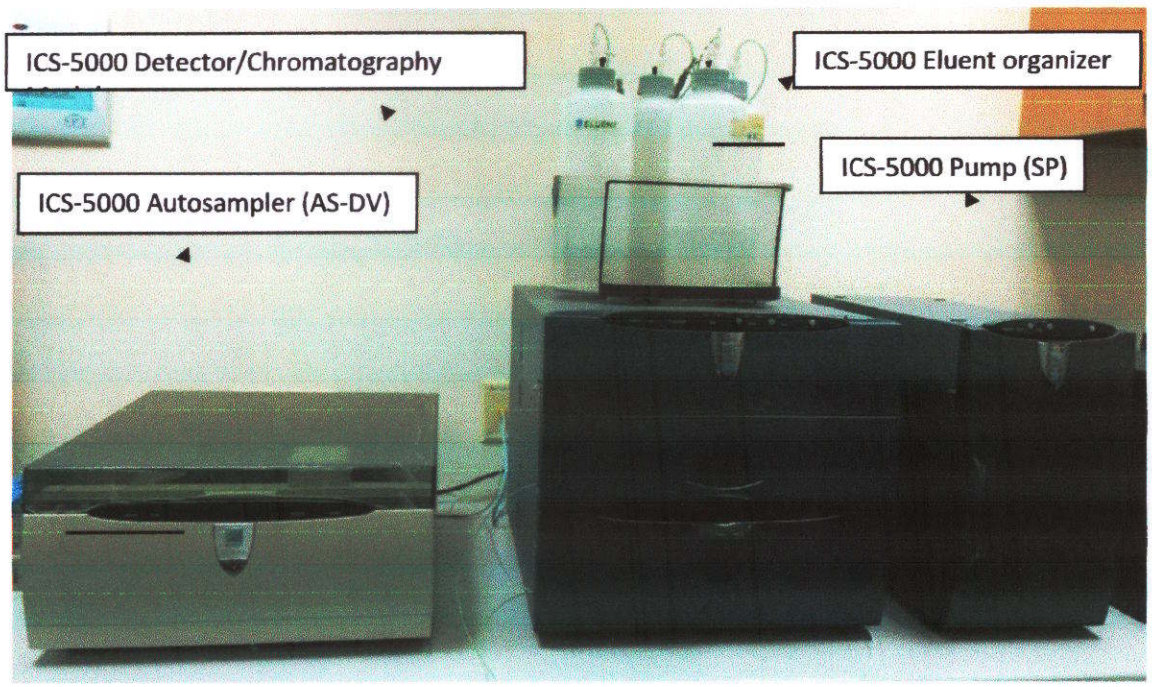
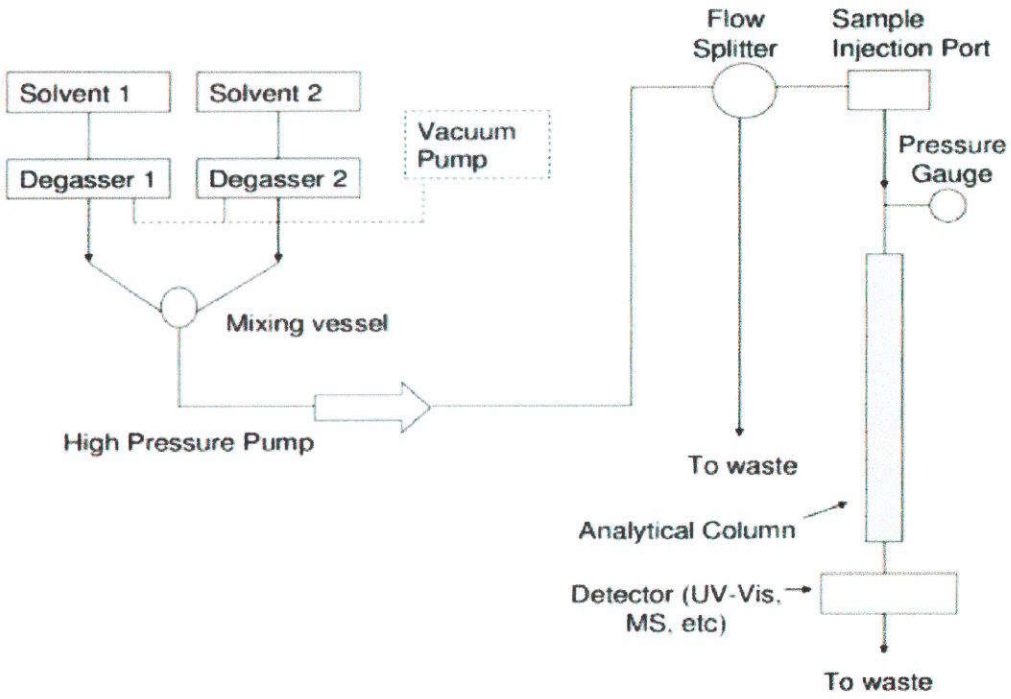
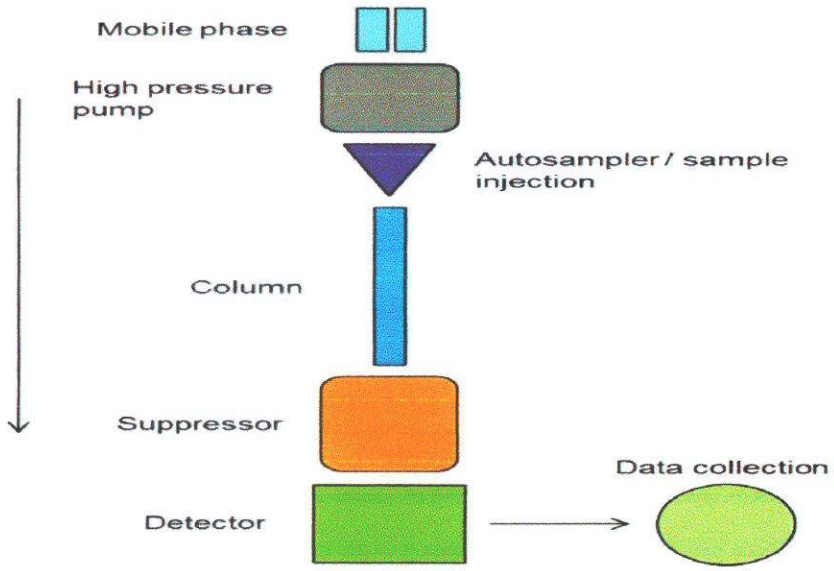


Figure15: Dionex ICS-5000 Ion Chromatography (IC) system Components in icddr,b Bangladesh.



16(a)



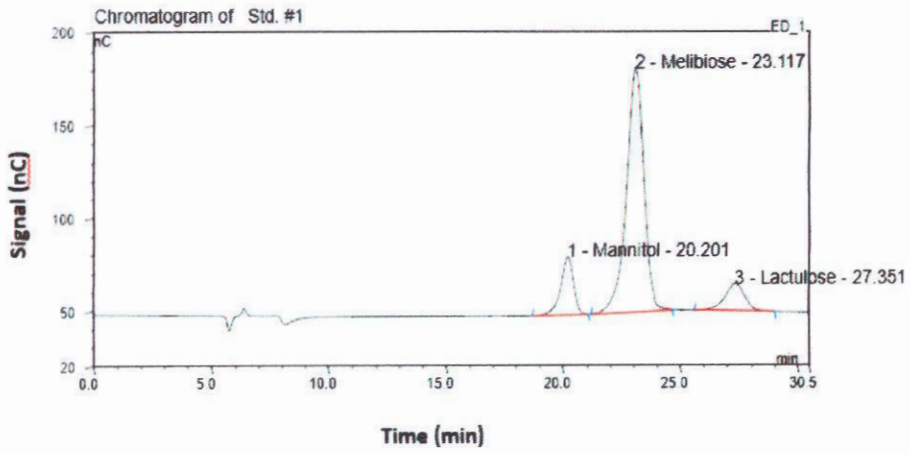
16(b)

Figure16. Schematic view of an HPIC instrumentation

Calculation of percent recovery of each sugar in the urine and their ratio:

Percent Lactulose /Mannitol recovery = [{total urine volume (dl) of 2 hours ×lactulose /Mannitol concentration (mg%) in urine} /Amount (mg) of lactulose /Mannitol taken] ×100

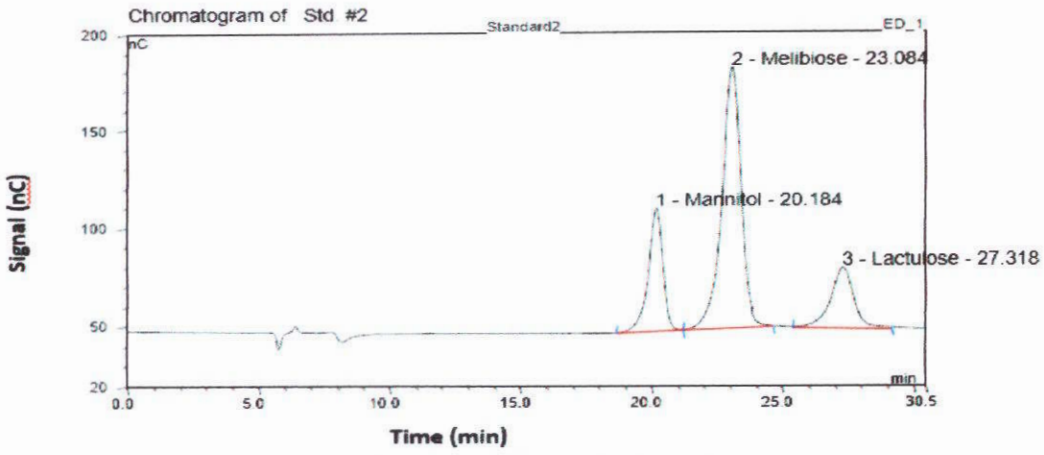
Chromatogram of Lactulose Mannitol & Melibiose (Standard01)



Lactulose	10 µM
Melibiose	50 µM
Mannitol	10 µM

17(a)

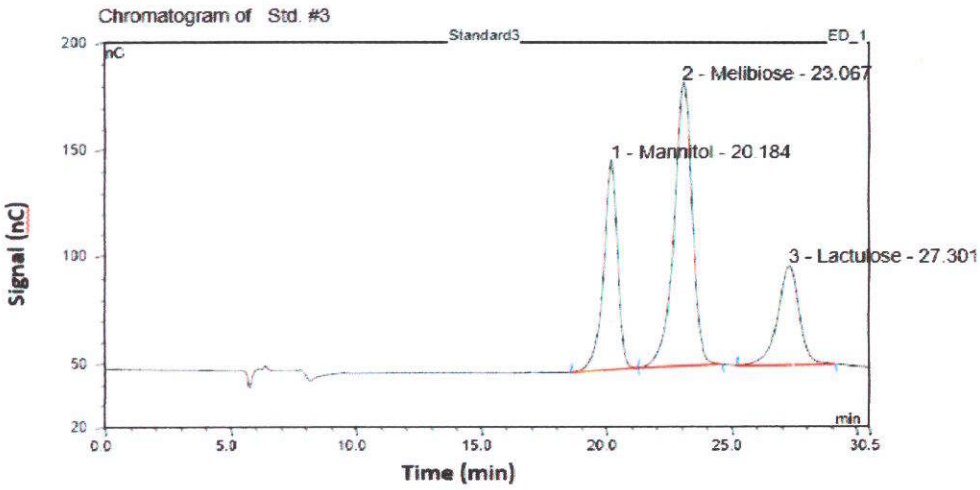
Chromatogram of Lactulose Mannitol & Melibiose (Standard02)



Lactulose	20 µM
Melibiose	50 µM
Mannitol	20 µM

17(b)

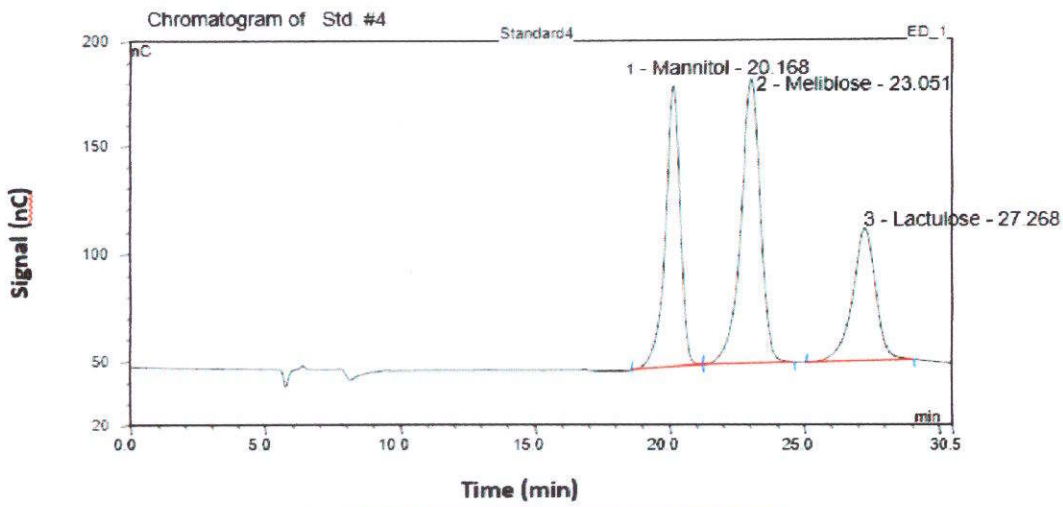
Chromatogram of Lactulose Mannitol & Melibiose (Standard03)



Lactulose	30 µM
Melibiose	50 µM
Mannitol	30 µM

17(c)

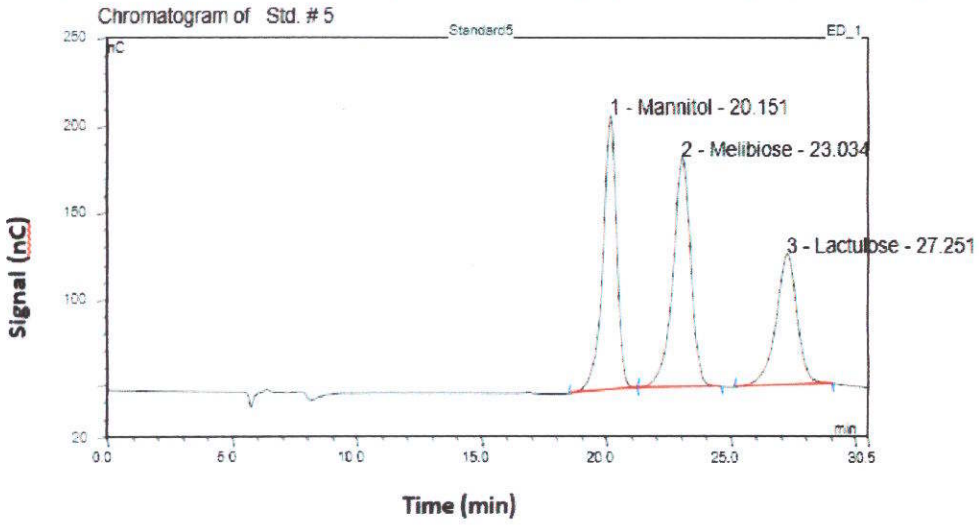
Chromatogram of Lactulose Mannitol & Melibiose (Standard04)



Lactulose	40 µM
Melibiose	50 µM
Mannitol	40 µM

17(d)

Chromatogram of Lactulose Mannitol & Melibiose (Standard05)

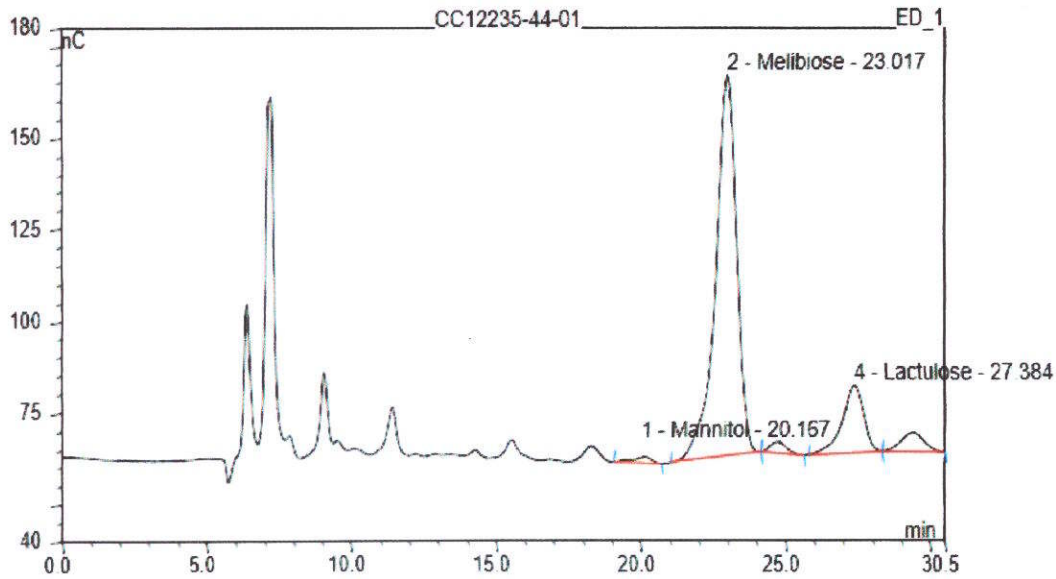


Lactulose	50 µM
Melibiose	50 µM
Mannitol	50 µM

17(E)

Chromatogram of urine specimen before lactulose mannitol solution intake:

Specimen ID_ CC1-2235-44-01(25 June,2016)

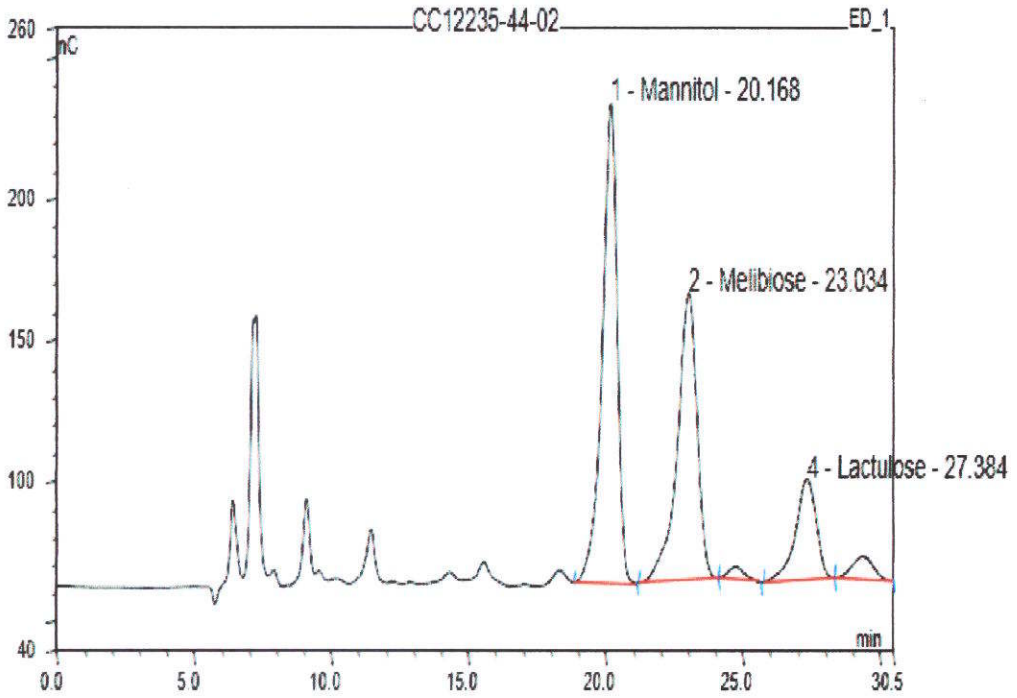


Lactulose	13.76 µM
Melibiose	48.83 µM
Mannitol	0.7865 µM

17(f)

Chromatogram of urine specimen after lactulose mannitol solution intake:

Specimen ID_ CC1-2235-44-02 (25 June,16)



Lactulose	27.47 µM
Melibiose	48.11 µM
Mannitol	68.58 µM

KU-IEC Acc, No- 4879, Date 15.9.2022

Figure 17 Chromatogram of Lactulose Mannitol and Melibiose standard 1,2,3,4,5 and Chromatogram of urine specimen before and after lactulose mannitol solution intake

ELISA (enzyme-linked immunosorbent assay) of C-reactive protein (CRP):

CRP is mainly formed in hepatocytes. The synthesis rate of crp is influenced by the cytokines involved in the inflammatory processes. The biological half-life time is estimated to be 13-16 hours. The serum crp concentration reflects very sensitive acute fever, pneumonia and myocardial infraction.

Recent studies describe an association between inflammatory reactions and cardiovascular diseases like arteriosclerosis or latent and chronic persist ent infections. As a marker for inflammation, **crp high-sensitive** can be used to predict the risk of myocardial infarction and stroke.

The crp determination in urine using the high sensitive elisa method allows - together with $\square_2$-macroglobulin - an early screening diagnose after kidney transplantations. The crp kit provides an easy-to-use assay for monitoring anti-rejection therapies. The elisa-assay was used for years to test hundreds of patients. Its predictive diagnostic value was compared with the gold s tandard (kidney biopsy).

Principle of the test

This enzyme immuno assay is a sandwich assay for the determination of crp in serum, plasma, urine and stool samples. The wells of the microtitre plate are coated with polyclonal antibodies directed against c-reactive protein. In a first incubation step, the crp in the samples is bound to the coated polyclonal rabbit antibodies (in excess). To remove all unbound substances, a washing step is carried out.

In a second incubation step, a peroxidase-labeled crp (po-antibody, polyclonal, rabbit-anti-crp) antibody is added. After another washing step, to remove all unbound substances, the solid phase is incubated with the substrate, tetramethylbenzidine (tmb). An acidic stopping solution is then added. The color converts to yellow. The intensity of the yellow color is directly proportional to the concentration of crp in the sample. A dose response curve of the absorbance (at 450 nm) unit vs. Concentration is generated. Crp, present in the patient samples, is determined directly from this calibration curve.

The combination of two specific antibodies in the crp elisa drastically reduces the possibility of wrong-negatives results and offers a secure diagnostic system to the user.

Material required for this Test:



- Ultra-pure water*
- Laboratory balance
- Precision pipettors calibrated and tips to deliver 10-1000 µl
- Covering foil for the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser

- Centrifuge capable of 3000 x g
- Vortex-mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm

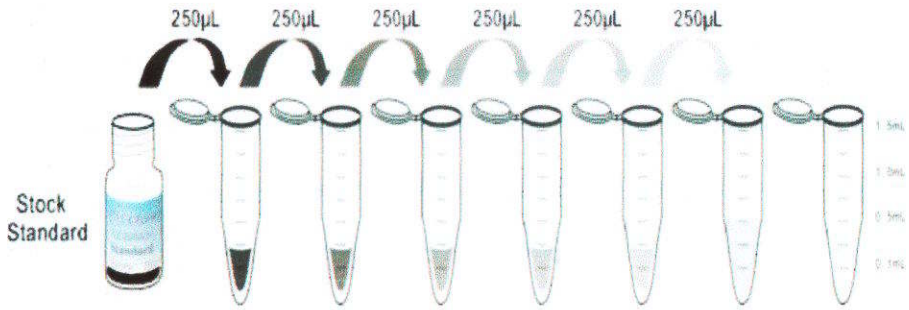
Specimen collection and preparation: I collected sufficient blood (at least 1 ml) by venipuncture into a tube or a plastic syringe, avoid hemolysis, centrifuge d for 15 minutes at 1,000 x g and 4°C and collect the serum.

Collection and storage of plasma: I Collected sufficient blood (at least 1 ml) by venipuncture into an EDTA venipuncture tube or a plastic syringe, centrifuge d for 15 minutes at 1,000 x g and 4°C within 10 minutes after blood collection and separate the plasma from the cells. Serum and plasma samples had to be diluted 1:100 or 1:500 before performing the assay. Then added 10 µl serum /plasma to 990 µl SAMPLEBUF (sample buffer), mixed well. (1:100) Used the dilution factor (100 or 500) to calculate the CRP concentration read off the calibration curve. Patient's samples with elevated CRP-concentrations must be diluted 1:4000 – 1:8000.

Reagent preparation:

1. Brought all kit components and samples to room temperature (18-25°C) before use. If the kit will not be used up

2. Standard - reconstituted the standard with 0.5ml of standard diluent, kept for 10 minutes at room Temperature, shaked gently (not to foam). The concentration of the standard in the stock solution is1,000pg/ml. prepared 7 tubes containing 0.25ml standard diluent and produce a double dilution Series according to the picture shown below. Mixed each tube thoroughly before the next transfer. Set up 7Points of diluted standard such as 1,000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml,15.6pg/ml, and the last ep tube with standard diluent was the blank as 0pg/ml.



pg/ml 1,000 500 250 125 62.5 31.2 15.6 0

3. Detection reagent a and detection reagent b - briefly centrifuged the stock detection a and Detection b before use. Diluted them to the working concentration 100-fold with assay diluent a and respectively.

4. **Wash Solution** - Diluted 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).

5. **TMB substrate** - Aspirated the needed dosage of the solution with sterilized tips .

Test procedure:

1. Prior to use in the assay allow all reagents and samples were placed in room temperature (18-26 °C) and mixed well.
2. The positions of STD (Standard) SAMPLE (Sample) CTRL (Controls) on a protocol sheet was labelled.
3. Microtiter strips out of the kit and unused strips were covered at 2-8° C.
3. Each well was washed 5 times by dispensing 250 µl of diluted wash buffer into each well. After the final washing step residual buffer was removed by tapping the plate on absorbent paper
5. 100 µl of STD (Standard) SAMPLE (Sample) CTRL (Controls) in duplicate into respective well was added.
6. The plate was tightly covered and 1 hour at room temperature (18-26°C) on a horizontal mixer was incubated the plate.
7. The contents of each well was discarded and Washed 5 times by dispensing 250 µl of diluted wash buffer into each well. After the final washing step residual buffer was removed by tapping the plate on absorbent paper.

8. 100 µl CONJ (conjugate) into each well was added.
9. The plate was tightly covered and incubated for 1 hour at room temperature (18-26°C) on a horizontal mixer.
10. The contents of each well was discarded and Washed 5 times by dispensing 250 µl of diluted wash buffer into each well. After the final washing step residual buffer was removed by tapping the plate on absorbent paper.
11. 100 µl of SUB (substrate) into each well was added.
12. Incubated for 10 - 20 minutes at room temperature (18-26°C) in the dark*
13. 50 µl of STOP (stop solution) was added into each well, mixed thoroughly.
14. Determined absorption immediately with an ELISA reader at 450 nm.

ELISA (enzyme-linked immunosorbent assay) of Soluble CD14:
Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human cd14 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any cd14 present is bound by the immobiliz ed antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human cd14 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of cd14 bound in the initial step. The color development is stopped and the intensity of the color is measured.

Materials

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 ml and 500 ml graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human cd14 controls (optional; r&d systems®, catalog # qc20).



Figure19: ELISA KIT OF Soluble CD14

Sample preparation

Serum and plasma samples required at least a 200-fold dilution prior to assay. A suggested 200-fold dilution is 10 µl sample + 1990 µl calibrator diluent rd5p (diluted 1:5)*.

Reagent preparation

Brought all reagents to room temperature before use.

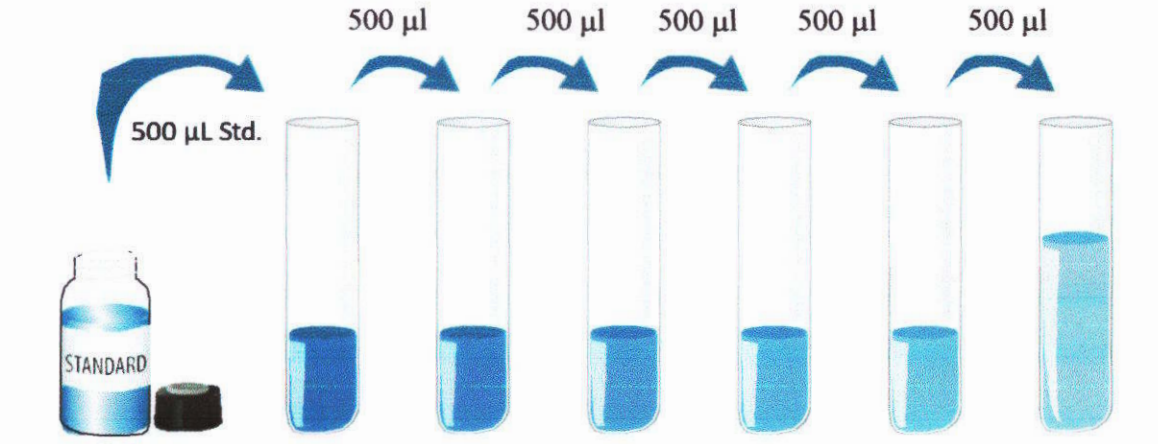
Wash buffer - Added 20 ml of wash buffer concentrate to deionized or distilled water to prepare 500 ml of wash buffer.

Substrate solution - color reagents a and b should be mixed together in equal volumes within 15 minutes of use.Protect from light. 200 µl of the resultant mixture was required per well.

Calibrator diluent rd5p (diluted 1:5) - added 20 ml of calibrator diluent rd5p concentrate to 80 ml of deionized or distilled water to prepare 100 ml of calibrator diluent rd5p (diluted 1:5).

Human cd14 standard – referred to the vial label for reconstitution volume. - reconstitute the human cd14 standard with calibrator diluent rd5p (diluted 1:5). This reconstitution produced a stock solution of 16,000 pg/ml. Allowed the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipetteed 500 µl of calibrator diluent rd5p (diluted 1:5) into each tube. Used the stock solution to produce a dilution series (below). Mixed each tube thoroughly before the next transfer. The undiluted human cd14 standard (16,000 pg/ml) served as the high standard. Calibrator diluent rd5p (diluted 1:5) served as the zero standard (0 pg/ml).



Test Procedure:

1. All reagents, research requirements, and samples were prepared.
2. Excess microplate stripes were removed from the plate frame, returned them to the desiccant packed foil pouch, and was resealed.
3. To each well 100 µL of Assay Diluent RD1W was added.
4. Standard, control, or sample* 100 µL per well was added and Cover ed with given adhesive stripe. Incubated to room temperature for 3 hours.

5. Aspired and washed each well, repeating the process for a total of four washes, three times.
6. 200 µL of Conjugate Human CD14 was applied to each well and Covered with fresh sticky board. Incubated to room temperature for 1 hour.
7. Aspired and washed each well, repeating the process for a total of four washes, three times.
8. To each well 200 µL of Substrate Solution was applied and Incubated above room temperature for 30 minutes.
9. 50 µL of Stop Solution into each well was inserted and The color of the wells was changed from blue to yellow
10. Determined the optical density within 30 minutes of each well, using a 450 nm microplate reader.



Figure20: Microplate reader used in calculating OD value.

Data analysis and calculation

For Windows, data was entered and analysed using SPSS (version 10.5; SPSS Inc., Chicago, IL, USA). Height-for -age-z-score (HAZ) and Weight-for-age z-score (WAZ) was calculated using the WHO growth standard (2010) http://www. who.int/childgrowth/en.

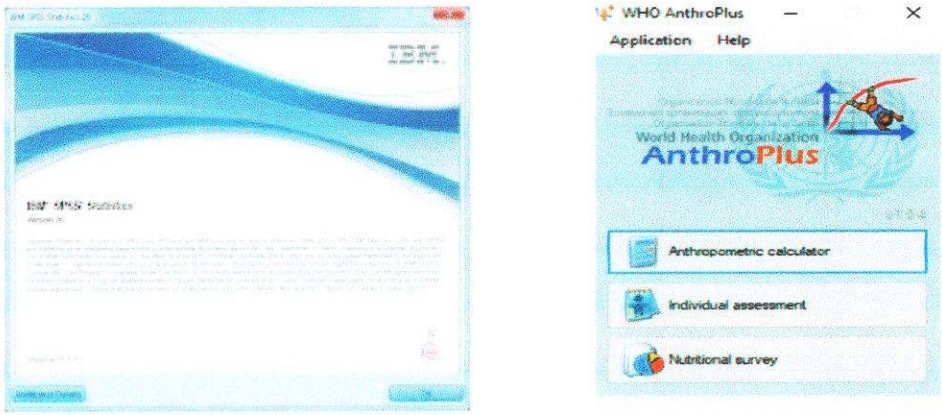


Figure21: SPSS (version 10.5; SPSS Inc., Chicago, IL, USA) and WHO Anthro plus

Chapter Four: Results and Analysis

Table 1: Male and Female percent

Sex	Frequency	Percent
Male	101	43.3
Female	132	56.7
Total	233	100.0

From Table 1 we described that among 233 subjects' the percent female was slightly more than male where 56.7% and 43.3% respectively. In this thesis data all subjects were randomly enrolled so there were no fixed criteria how many male or female will be enrolled. As enrolled number was higher in the female group so their percent was higher.

Table 2: Percent of Male and Female among Stunted, at risk of stunting and Healthy control

	Frequency	**Percent**	**Male**	**Female**
Stunted	**54**	**23.2**	**21(38.88%)**	**33(61.11%)**
At risk of stunting	**101**	**43.3**	**50(49.50%)**	**51(50.49%)**
Healthy control	**78**	**33.5**	**30(38.46%)**	**48(61.53%)**
Total	**233**	**100.0**	**101**	**132**

From Table 2 we summarized that according to who standard growth chart <-2 of Height for age Z score they will be dedicated as stunted .From this counting we could say that the number of stunted children were 54 and they reached just above 23%, where male comparatively 20 % less than female but female number was high .The reason behind female percent may be their higher number where male 38.88% and female 61.11% respectively. Female stunting had may be many reasons like their diet habit, hygiene practice and their biological and genetica lly makeup.

In group at risk of stunting HAZ (height for age Z score) was -1 to-2. In this team both male and female percent was near about same but female were more healthy(61.53%) as they had HAZ (Height for Z score was -1<) where male were nearly 10% less than female .
Finally from this above table number 2 we concluded that among 233 subjects more than eighty percent of people were not severely malnourished where female number was high and four times less than the number of relatively well nourished who had good linear growth.

Table 3: EED by measuring L:M ratio at 52 weeks of children

EED		**Normal**	
Male%	**Female%**	**Male %**	**Female%**

75(47.46%)	83(52.53%)	26(34.66%)	49(65.33%)
Total=158 (67.81%)		Total=75 (32.18%)	

In the above-mentioned table 3 it concluded that among 233 subjects over 65% had the elevated presence of lactulose, mannitol ratio EED as they lactulose mannitol ratio more than 0.07 which was our cut off value at 2 hours whereas only 32% had normal gut barr ier. As a result this nearly 70% infants had the greatest risk of being stunted, malnourished and poor neurocognitive development. Besides we considered it as our gold standard diagnostic method of determining environmental enteric dysfunction as it was no invasive biomarker to diagnose the EED as like many researcher who used it as a gold standard method for EED diagnosis among infants special under 2 years. Among this female were more than 50% and male were near about 50%.from this percent we could tell t aht may be female gut permeability due environmental exposures like pathogens, fecal matters and others key substances.

Besides this almost 33% were not affected by environmental factors who were responsible for causing this complex pathogenesis where fem ale had more normal gut barrier bearing 65.33% while male had nearly half of them.
From this table we told that according to table value we female had greater chance to get affected by gut barrier dysfunction.

Table 4: EED due to CRP at 52 weeks of children

EED			Normal	
Male %	Female%		Male%	Female%
23 (44.23%)	29(55.76%)		78(43.09%)	103(56.90%)
Total=52 (22.31%)			Total=181 (77.68%)	

In the above-mentioned table 4 we concluded that for the chronic systemic inflammation there were higher presence of CRP non –invasive biomarker among 233 subjects 52 were EED and their percentage around 23 where we used cut off value was 5mg/l where female were more EED symptoms than male subjects .Among 52 abnormal enteric structure 29 female were EED and 10% less than in male subjects where 55.76% and 44.23% respectively. In comparison with gold standard methods CRP biomarkers had 3times less than lactuolse ,mannitol ratio EED compare to EED due to dual sugar test.

Secondly, In case of prevalence of normal gut barrier due to inflammatory marker CRP had more than gold standard about 2 times where female were more than male in case of intact gut barrier who were not exposed with environment where 78 subjects had normal gut and their female counterpart had 103 whereas 43.09% and 56.90% respectively.

Table5: EED due to sCD14 at 52 weeks of children

EED			Normal	
Male %	Female%		Male%	Female%
88 (44.4%)	110(55.5%)		13(37.14%)	22(57.89%)

Total=198 (84.97%)	Total=35 (15.02%)

Table 5 was depicted that prevalence of EED among 233 subjects for the presence of soluble CD14 in the serum close to 85% where cut off value was 1450ng/ml and there were bacterial trans location most probably confirmed and for that reason number of EED subjects were nearly 200 and male were only 45% whereas female were around 56% and here female were environmental enteric dysfunction about 10% percent higher than male. By comparing gold standard result of EED it had crossed that number where around 67% was EED in L:M ratio perspective but here in close to85% showed EED symptoms.

In case of normal gut only 15 % showed normal gut activity as there were no presence of soluble CD14 in the blood samples greater than 1450ng/ml and there may be no bacterial translocation happened .Among 35 subjects female were nearly two times higher than male bearing 22 and 13 respectively and their percentage was 57.89% and 37.14%.

Table 6: Descriptive Analysis by Nutritional status Group

		Height for Age Z score	Level of CRP in mg/l	Level of sCD14 in ng/ml	Lactulose-Mannitol Ratio
		Mean	Mean	Mean	Mean
Nutritional Status	Stunting	-2.66	5.65	1971.24	1.71
	At risk of stunting	-1.46	4.52	1964.23	0.43
	Healthy control	-.34	5.81	1948.15	0.31

From this above table 6 we described that HAZ (height for age Z score) mean of stunted group was -2.66 as we considered stunted who had HAZ score <-2 as a result their mean was close to that value which increased at risk group nearly one in perspective of mean and like before in a healthy control group they had increased their mean value nearly 1.

In case of CRP stunted group had higher mean value as we knew that due to gut permeability there were systemic inflammation occurred and the CRP value was higher in the blood but in case of healthy control group had more CRP value than stunted what is the reason b ehind this biology i do not know but these issues may me researched din a planed way. Beside these mean of CRP value was lower than stunted which was correlated with my thesis hypothesis and also less than healthy control group which was out of my knowledge what is reason behind it.

In addition, sCD14 had the highest mean value in stunted group compare to other two groups which was similar to our hypothesis as we considered that stunted subjects will have high systemic inflammatory biomarkers value in the blood .The level of scd14 had decreased from stunting to at risk group and healthy group.

Finally, in case of L:M ratio we summarized that stunted had highest mean like sCD14 and there was good indicator of gut barrier dysfunction was proved as we conside red it as a gold standard method for EED diagnosis among children under 2 years of age.

In conclusion we could say that due to gut permeability level of scd14 and CRP mean was increased which we considered EED by L:M ratio.

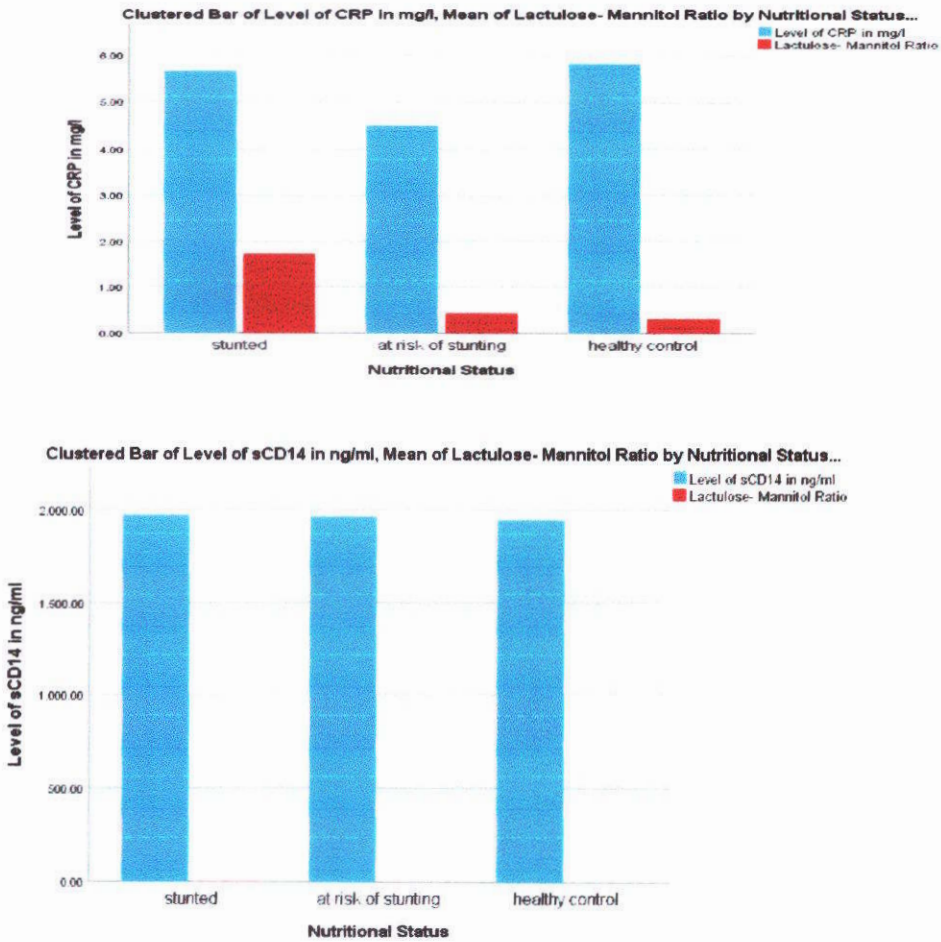


Figure 22: Level of three non-invasive biomarkers of nutritional status group.

From this bar graph In case of CRP stunted group had higher mean value as we knew that due to gut permeability there were systemic inflammation occurred and the CRP value was higher

in the blood but in case of healthy control group had more CRP value than stunted what is the reason behind this biology i do not know but these issues may me researched din a planed way. Beside these mean of CRP value was lower than stunted which was correlated with my thesis hypothesis and also less than healthy control group which was out of my knowledge what is reason behind it.

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Finally, in case of L:M ratio we summarized that stunted had highest mean like sCD14 and there was good indicator of gut barrier dysfunction was proved as we considered it as a gold standard method for EED diagnosis among children under 2 years of age.

In conclusion we could say that due to gut permeability level of scd14 and CRP mean wa s increased which we considered EED by L:M ratio.

Table 7: Mann-Whitney Test for Stunted vs At risk of stunting

Parameter	p-value (95% CI)
sCD14	.838
CRP	.269
LM ratio	.108

From Table 7 we could write that stunted group and at risk of stunting group had non-significant correlation in case of bacterial translocation biomarkers soluble CD14 bearing p-value (95%CI) was 0.838 .As a result we can say that there was correlation which was statistically non-significant.

In case of CRP there was less non-significant result with respect to soluble CD14 in stunted and at risk of stunting group bearing p-value (95%CI) was .269 which signified that there were no significant correlation for systemic inflammatory biomarkers CRP between stunted and at risk of stunting group.

Finally, lactulose and mannitol ratio was most less non -significant with respect to other two biomarkers bearing p-value (95%CI) was .108 which mentioned that there was no significant correlation of gut barrier dysfunction biomarkers (L:M ratio) between stunted and at risk of stunting group.

Table 8: Mann-Whitney Test for Stunted vs Healthy

Parameter	p-value (95% Cl)
sCD14	0.993
CRP	0.701
LM ratio	0.220

From Table 8 we could write that stunted group and healthy group had non-significant correlation in case of bacterial translocation biomarkers soluble CD14 bearing p-value (95%CI) was 0.993 .As a result we can say that there was correlation which was statistically non-significant.

In case of CRP there was less non-significant result with respect to soluble CD14 in stunted and healthy group bearing p-value (95%CI) was .701 which signified that there were no significant correlation for systemic inflammatory biomarkers CRP between stunted and healthy group.

Finally, lactulose and mannitol ratio was most less non-significant with respect to other two biomarkers bearing p-value (95%CI) was .220 which mentioned that there was no significant correlation of gut barrier dysfunction biomarkers (L:M ratio) between stunted and healthy group.

Table 9: Mann-Whitney Test for Healthy vs At risk of stunting

Parameter	p-value (95% Cl)
sCD14	0.911
CRP	0.593
LM ratio	0.771

From Table 9 we could write that at risk of stunting group and healthy group had non-significant correlation in case of bacterial translocation biomarkers soluble CD14 bearing p-value (95%CI) was 0.991 .As a result we can say that there was correlation which was statistically non-significant.

In case of CRP there was less non-significant result with respect to soluble CD14 between at risk of stunting and healthy group bearing p-value (95%CI) was .593 which signified that there were no significant correlation for systemic inflammatory biomarkers CRP between at rsik of stunting and healthy group.

Finally, lactulose and mannitol ratio was most less non-significant with respect to other two biomarkers bearing p-value (95%CI) was .771 which mentioned that there was no significant correlation of gut barrier dysfunction biomarkers (L:M ratio) between at risk of stunting and healthy group.

Table 10: Descriptive Statistics of Four Parameters			
	N	Mean	Std. Deviation
Height for Age Z score	233	-1.3652	.95854
Level of sCD14 in ng/ml	233	1960.4730	499.52460
Level of CRP in mg/l	233	5.2119	12.56729
Lactulose- Mannitol Ratio	233	.6876	4.66472
Valid N (listwise)	233		

From this descriptive statistics table 10 we summarized that HAZ (Height for Age Z score) mean of 233 subjects was -1.36 and its SD (Standard Deviation) was 0.95 and there was small standard deviation and signified that maximum value were close to the central tendency

Besides level of sCD14 mean of 233 subjects was 1960 in ng/ml and it's SD (standard deviation) close to 500 that meant that there was high standard deviation from the mean value and the many values were deviated from the central tendency.

In case of level of CRP the mean of 233 subjects was above 5 and it's SD just above 12 and there was high label of standard deviation was present among the mean value of the subjects.

In case of parameter L:M ratio mean was 0.68 and its SD was 4.66 and there was also high label of standard deviation happened among the 233 subjects L:M ratio mean value and also told that small number of value were present in the central tendency point .

Table 11: Correlations Among four parameters

		Level of CRP in mg/l	Level of sCD14 in ng/ml	Height for Age Z score	Lactulose-Mannitol Ratio
Level of CRP in mg/l	Pearson Correlation	1	.242**	-.028	-.009
	Sig. (2-tailed)		.000	.666	.891
	N	233	233	233	233
Level of sCD14 in ng/ml	Pearson Correlation	.242**	1	-.015	-.059
	Sig. (2-tailed)	.000		.822	.372
	N	233	233	233	233
Height for Age Z score	Pearson Correlation	-.028	-.015	1	-.138*
	Sig. (2-tailed)	.666	.822		.035
	N	233	233	233	233

Lactulose-Mannitol Ratio	**Pearson Correlation**	**-.009**	**-.059**	**-.138***	**1**
	Sig. (2-tailed)	**.891**	**.372**	**.035**	
	N	**233**	**233**	**233**	**233**
****. Correlation is significant at the 0.01 level (2-tailed).**					
***. Correlation is significant at the 0.05 level (2-tailed).**					

From this correlations table 11 we observed that level of CRP had positive Pearson correlation with sCD14 bearing r value 0.242 and there was significant correlation present bearing p value .000 and we also summarized that if the inflammatory biomarkers CRP level will increase the level of bacterial translocation biomarkers soluble CD14 also increased in th e blood and there was significant correlation between the systemic inflammatory biomarkers.

In case of HAZ (height for age Z score) with level of CRP value in the 233 subjects there was negative correlation bearing pearson correlation value r=-.028 which signified that the increasement of CRP in the blood the height of that subjects was retarded but this correlation was non-significant as their p –value was .66 which meant that though there was correlation but that was not much significant .

Finally correlation between level of CRP in the blood and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r=-.009 which meant that the gut barrier disruption among the subjects not show the high lev el of inflammation and this correlation was statistically non-significant as they had p-value .891 at the level of 0.05 (2-tailed).

Level of soluble CD14 had positive Pearson correlation with CRP bearing r value 0.242 and there was significant correlation present bearing p value .000 and we also summarized that if the inflammatory biomarkers CRP level will increase the level of bacterial translocation biomarkers soluble CD14 also increased in the blood and there was significant correlation between the systemic inflammatory biomarkers.

In case of HAZ (height for age Z score) with level of sCD14 value in the 233 subjects there was negative correlation bearing pearson correlation value r=-.015 which signified that the increasement of Scd14 in the blood the height of that subjects was retarded but this correlation was non-significant as their p –value was .822 which meant that though there was correlation but that was not much significant .

Finally correlation between level of soluble CD14 in the blood and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r= -.059 which meant that the gut barrier disruption among the subjects not show th e high level of bacterial translocation and systemic inflammation and this correlation was statistically non-significant as they had p-value .372 at the level of 0.05 (2-tailed).

HAZ (height for age Z score) had negative Pearson correlation with CRP bearing r value 0.028 and there was non-significant correlation present bearing p value .666 and we also

summarized that the inflammatory biomarkers CRP level will increase if the HAZ value decrease and there was non-significant correlation between the systemic inflammatory biomarkers and HAZ.

In case of HAZ (height for age Z score) with level of sCD14 value in the 233 subjects there was negative correlation bearing pearson correlation value r=-.015 which signified that the increasement of Scd14 in the blood the height of that subjects was retarded but this correlation was non-significant as their p –value was .822 which meant that though there was correlation but that was not much significant .

Finally correlation between HAZ (height for age Z score) and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r=-.138 which meant that the gut barrier disruption among the subjects show the high level of HAZ score which means that if the gut barrier disruption occur then the height of that subjects will sluggish and this correlation was statistically non-significant as they had p-value .035 at the level of 0.05 (2-tailed).

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Here in correlation between level of CRP in the blood and gut permeability bio markers lactulose mannitol raito was few negative bearing pearson correlation value r=-.009 which meant that the gut barrier disruption among the subjects not show the high level of inflammation and this correlation was statistically non-significant as they had p-value .891 at the level of 0.05 (2-tailed).

Correlation between level of soluble CD14 in the blood and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r=-.059 which meant that the gut barrier disruption among the subjects not show the high level of bacterial translocation and systemic inflammation and this correlation was statistically non-significant as they had p-value .372 at the level of 0.05 (2-tailed).

In conclusion, correlation between HAZ (height for age Z score) and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r=-.138 which meant that the gut barrier disruption among the subjects show the high level of HAZ score which means that if the gut barrier disruption occur then the height of that subjects will sluggish and this correlation was statistically non-significant as they had p-value .035 at the level of 0.05 (2-tailed).

Relationship between Lactulose Mannitol ratio (gut permeabiality biomarker) with Stunting:

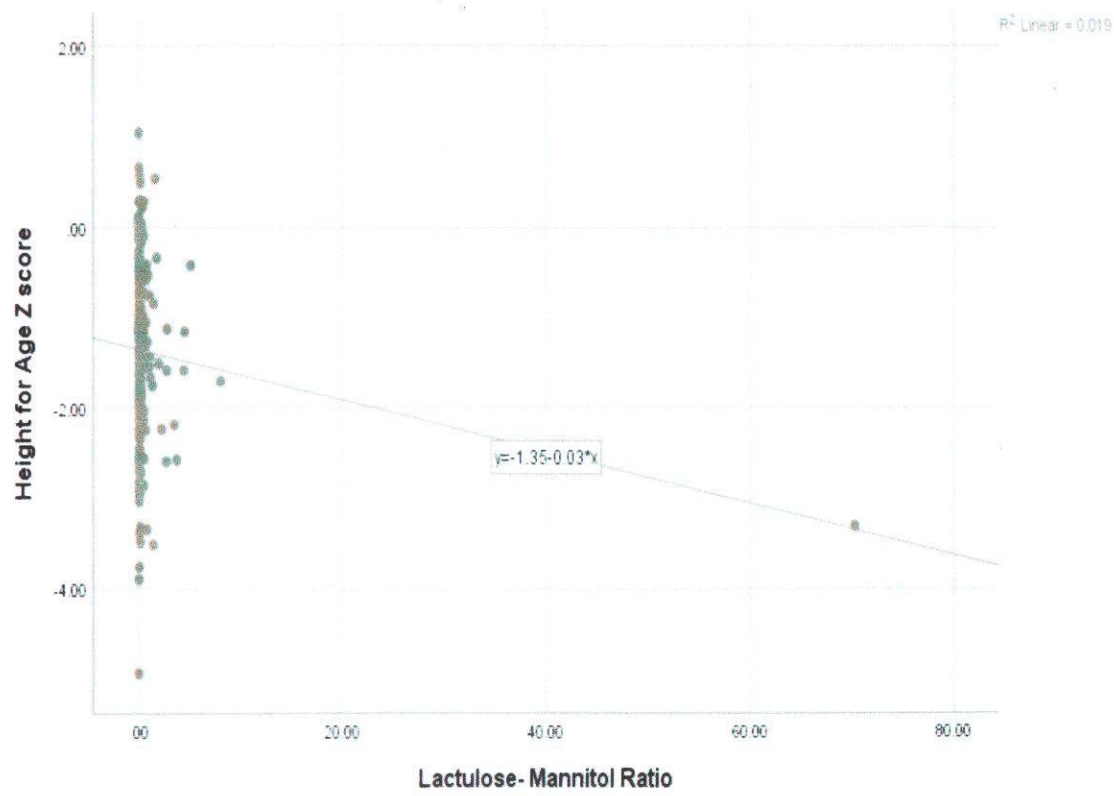


Figure 23: Height for Age Z score and Lactulose -Mannitol Ratio

From figure 23we summarized that correlation between HAZ (height for age Z score) and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r=-.138 which meant that the gut barrier disruption among the subjects show the high level of HAZ score which means that if the gut barrier disruption occur then the height of that subjects will sluggish and this correlation was statistically non-significant as they had p-value .035 at the level of 0.05 (2-tailed).

Relationship between Soluble CD14 (microbial translocation biomarker) with stunting:

KHULNA UNIVERSITY Central Library

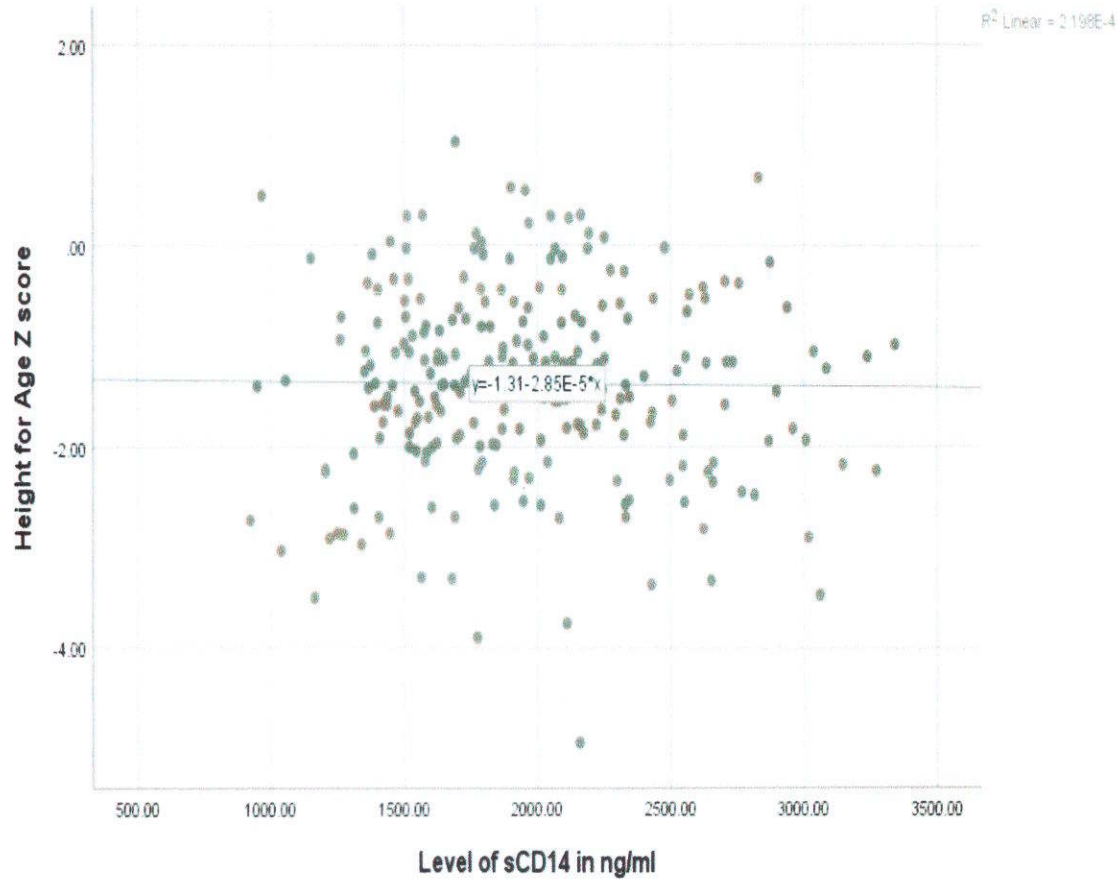


Figure 24: Height for Age Z score and Level of sCD14 in ng/ml

From this figure24 we concluded that In case of HAZ (height for age Z score) with level of sCD14 value in the 233 subjects there was negative correlation bearing pearson correlation value r=-.015 which signified that the increasement of Scd14 in the blood the height of that subjects was retarded but this correlation was non-significant as their p –value was .822 which meant that though there was correlation but that was not much significant .

Relationship between C-Reactive protein (inflammatory biomarker) with stunting:

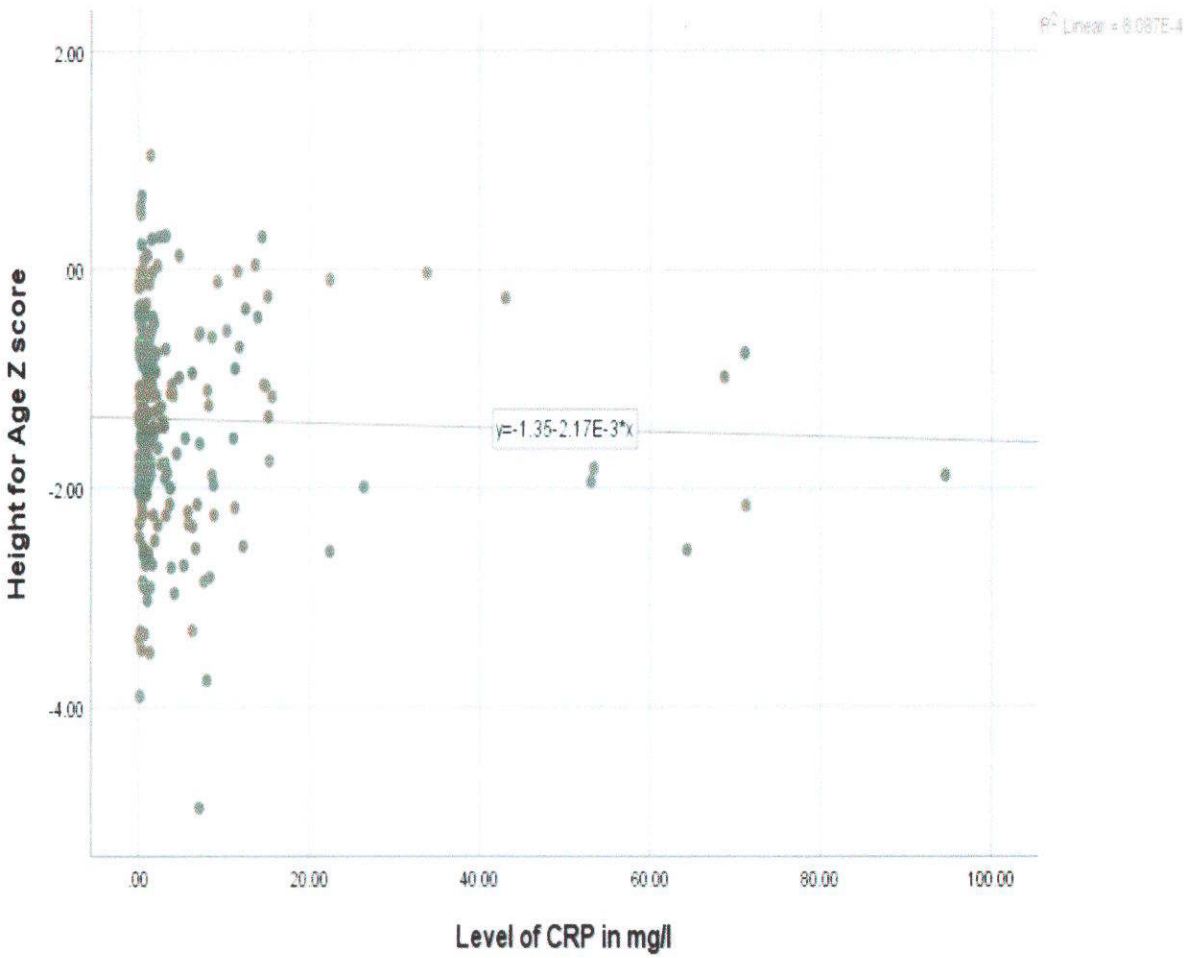


Figure 25: Height for Age Z score and Level of CRP in mg/ml

From this figure 25 we concluded that HAZ (height for age Z score) had negative Pearson correlation with CRP bearing r value- 0.028 and there was non-significant correlation present bearing p value .666 and we also summarized that the inflammatory biomarkers CRP level will increase if the HAZ value decrease and there was non-significant correlation between the systemic inflammatory biomarkers and HAZ.

Internal relationship among three non-invasive Biomarkers:

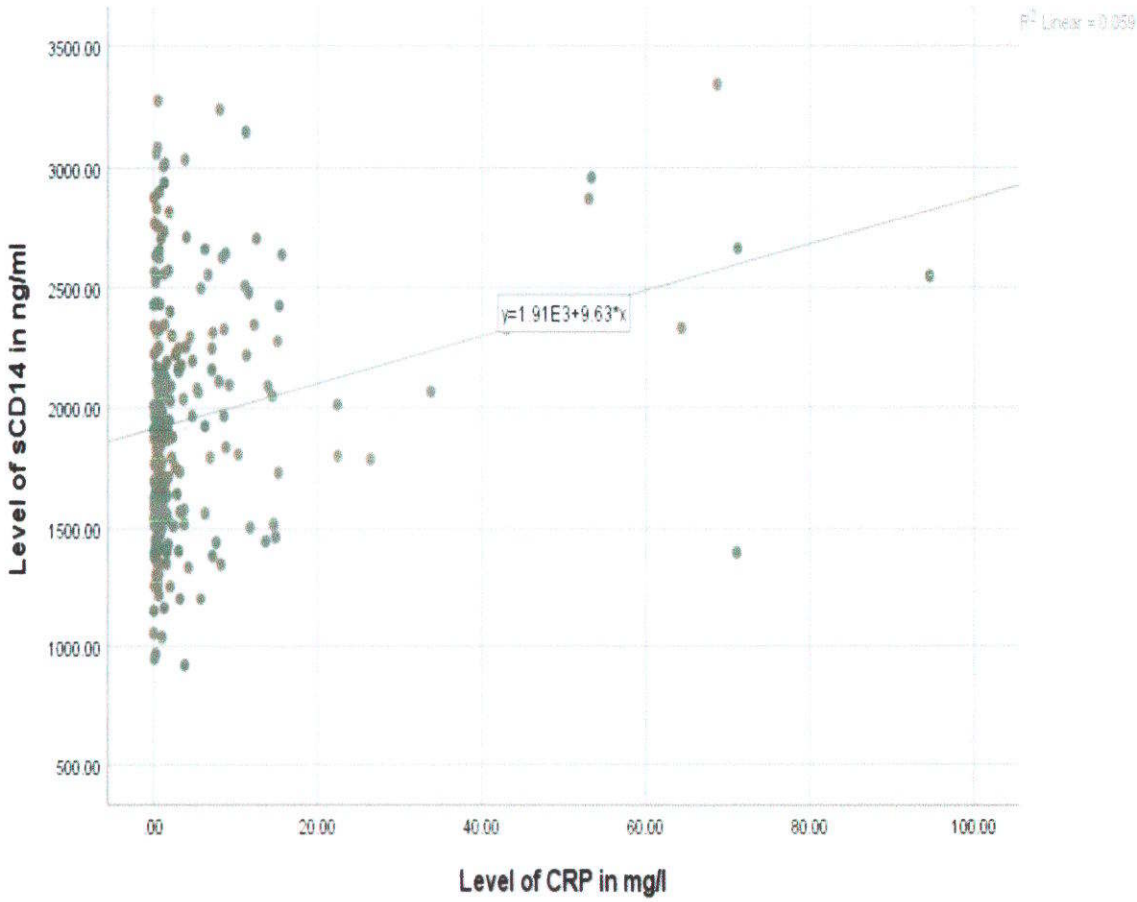


Figure 26: Level of sCD14 in ng/ml and Level of CRP in mg/ml

From this figure 26 we told that level of CRP had positive Pearson correlation with sCD14 bearing r value 0.242 and there was significant correlation present bearing p value .000 and we also summarized that if the inflammatory biomarkers CRP level will increase the level of bacterial translocation biomarkers soluble CD14 also increased in the blood and there was significant correlation between the systemic inflammatory biomarkers.

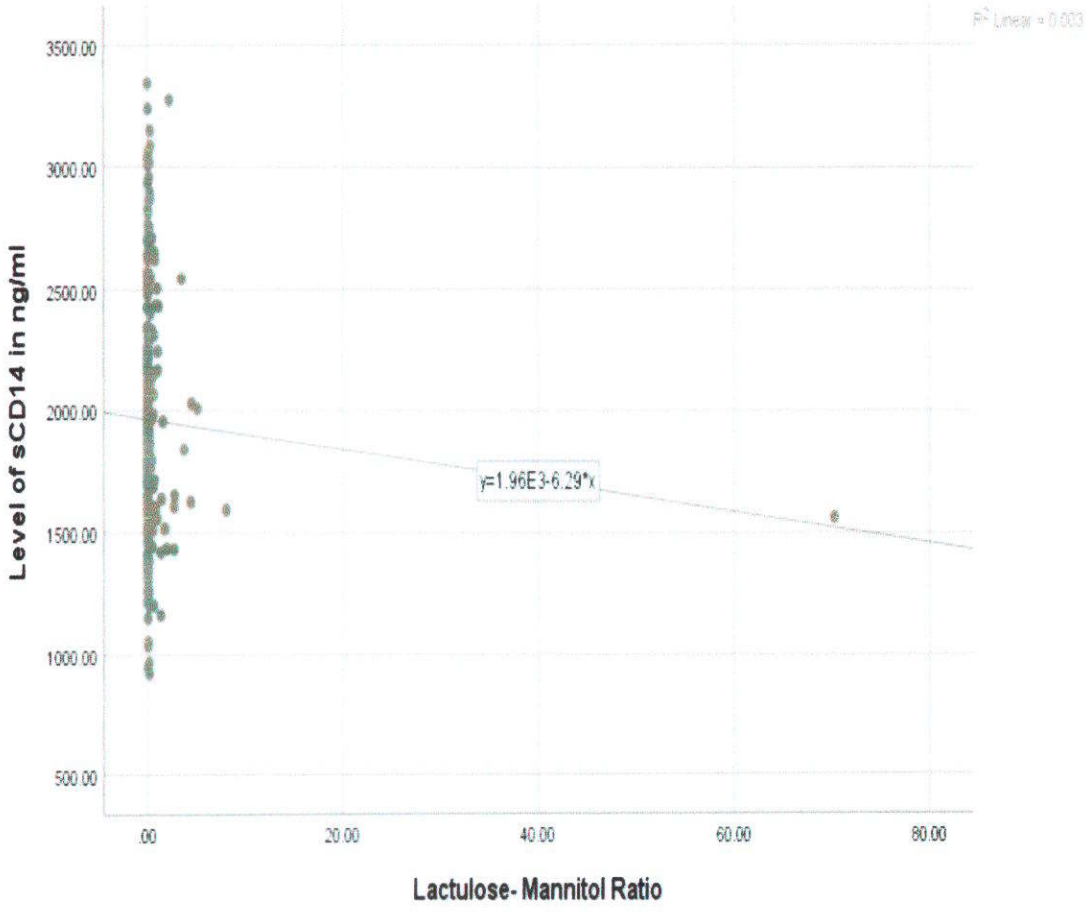


Figure 27: Level of sCD14 in ng/ml and Lactulose -Mannitol Ratio

From this figure 27 we told that correlation between level of soluble CD14 in the blood and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r=-.059 which meant that the gut barrier disruption among the subjects not show the high level of bacterial translocation and systemic inflammation and this correlation was statistically non-significant as they had p-value .372 at the level of 0.05 (2-tailed).

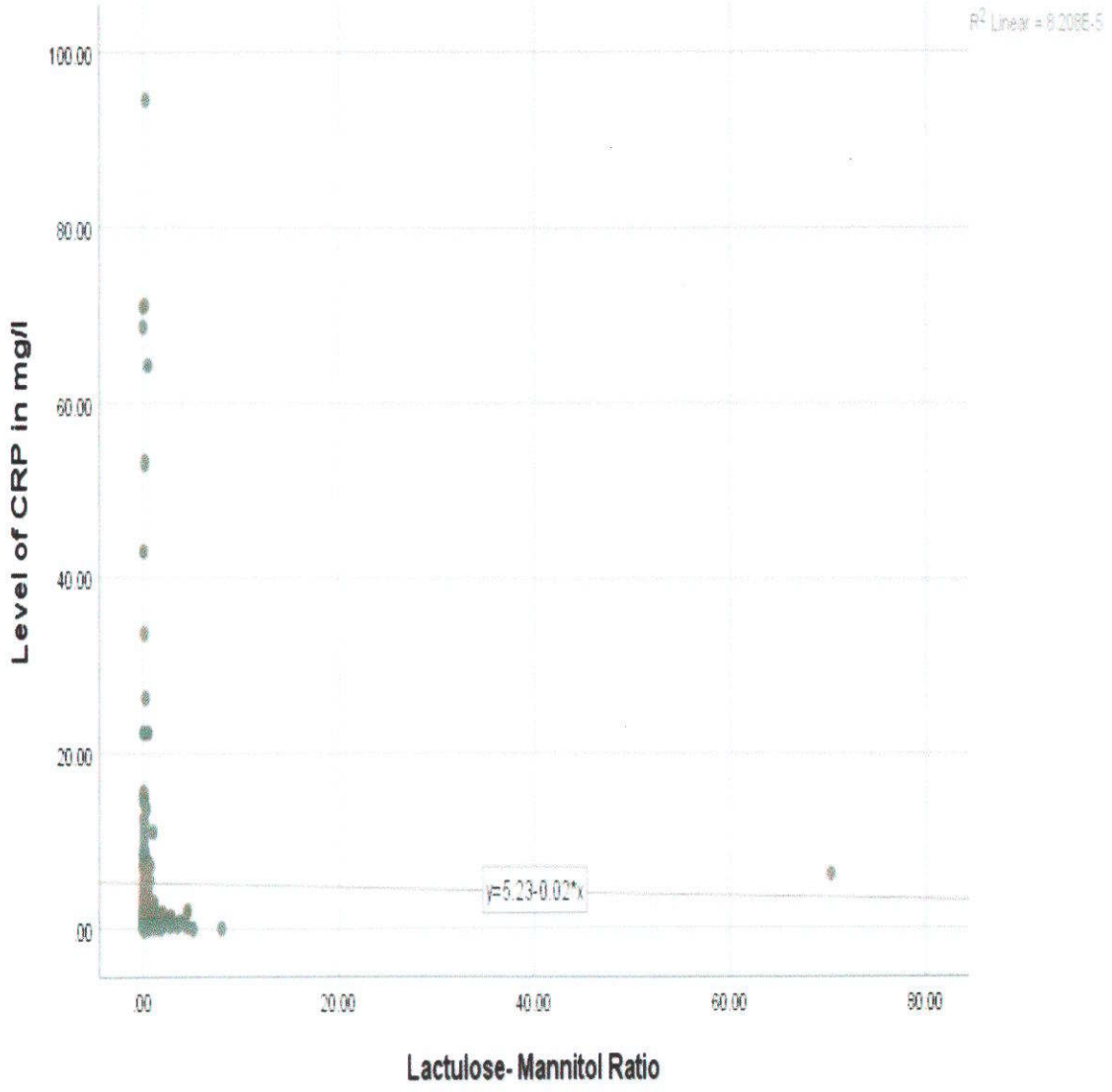


Figure 28: Level of CRP in mg/ml and Lactulose -Mannitol Ratio

From this figure 28 we told correlation between level of CRP in the blood and gut permeability biomarkers lactulose mannitol raito was few negative bearing pearson correlation value r= -.009 which meant that the gut barrier disruption amon g the subjects not show the high level of inflammation and this correlation was statistically non-significant as they had p-value .891 at the level of 0.05 (2-tailed).

Table12 Group Statistics of four parameters between stunting and at risk of stunting

	Nutritional Status	N	Mean	Std. Deviation
Lactulose- Mannitol Ratio	stunted	54	1.7147	9.53975
	at risk of stunting	101	.4307	1.08155
Height for Age Z score	stunted	54	-2.6559	.56493
	at risk of stunting	101	-1.4637	.29077
Level of sCD14 in ng/ml	stunted	54	1971.2378	604.47950
	at risk of stunting	101	1964.2329	475.07054
Level of CRP in mg/l	stunted	54	5.6457	12.98760
	at risk of stunting	101	4.5194	12.27920

From above table 12 we could summarize that mean of L:M ratio between two nutritional status stunted and at risk of stunting group had more than 1 mean difference as they had mean value 1.71 and 0.43 respectively which meant that gut barrier disruption in stunted group was present. Besides, HAZ (height for age z score) between stunting and at risk of stunting group had mean of HAZ -2.65 and -1.4 respectively. In case of level of soluble CD14 between stunted and at risk of stunting group had high in stunted group bearing 1971ng/ml where at risk group taking mean 1964ng/ml and from this we could say that in the blood level of soluble CD14 was high in the stunted group. Finally the level of inflammatory biomarker CRP was higher in stunted group with respect to at risk group bearing mean 5.64mg/l and 4.51mg/l the cause behind this inflammation in the body in stunted group was high.

Table13 Group Statistics of four parameters between at risk of stunting and healthy control

	Nutritional Status	N	Mean	Std. Deviation
Lactulose- Mannitol Ratio	stunted	54	1.7147	9.53975
	healthy control	78	.3091	.65542
Height for Age Z score	stunted	54	-2.6559	.56493
	healthy control	78	-.3441	.44902
Level of sCD14 in ng/ml	stunted	54	1971.2378	604.47950
	healthy control	78	1948.1520	454.91645

Level of CRP in mg/l	stunted	54	5.6457	12.98760
	healthy control	78	5.8084	12.76012

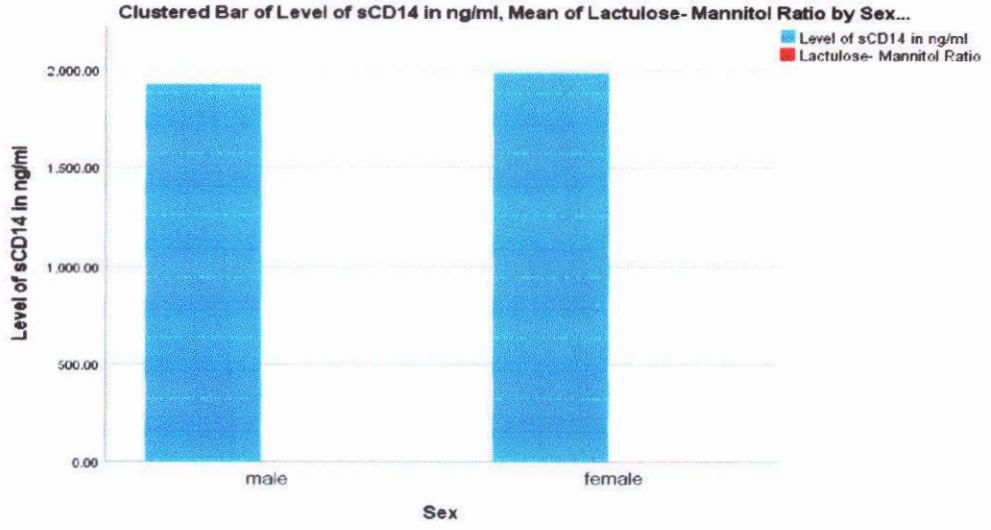
From above table From above table 12 we could summarize that mean of L:M ratio between two nutritional status stunted and healthy group had more than 1 mean difference as they had mean value 1.71 and 0.30 respectively which meant that gut barrier disruption in stunted group was present. Besides, HAZ (height for age z score) between stunting and healthy group had mean of HAZ -2.65 and -.34 respectively. In case of level of soluble CD14 between stunted and healthy group had high in stunted group bearing 1971ng/ml where at risk group taking mean 1948 ng/ml and from this we could say that in the blood level of soluble CD14 was high in the stunted group. Finally the level of inflammatory biomarker CRP in stunted group had less than healthy group bearing mean 5.64mg/l and 5.80mg/l.

Table14 Group Statistics of four parameters between at risk of stunting and healthy control

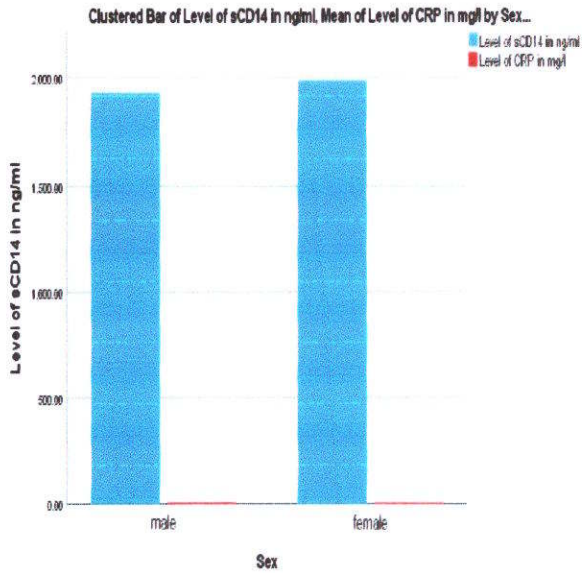
	Nutritional Status	N	Mean	Std. Deviation	Std. Error Mean
Lactulose- Mannitol Ratio	at risk of stunting	101	.4307	1.08155	.10762
	healthy control	78	.3091	.65542	.07421
Height for Age Z score	at risk of stunting	101	-1.4637	.29077	.02893
	healthy control	78	-.3441	.44902	.05084
Level of sCD14 in ng/ml	at risk of stunting	101	1964.2329	475.07054	47.27129
	healthy control	78	1948.1520	454.91645	51.50915
Level of CRP in mg/l	at risk of stunting	101	4.5194	12.27920	1.22183
	healthy control	78	5.8084	12.76012	1.44480

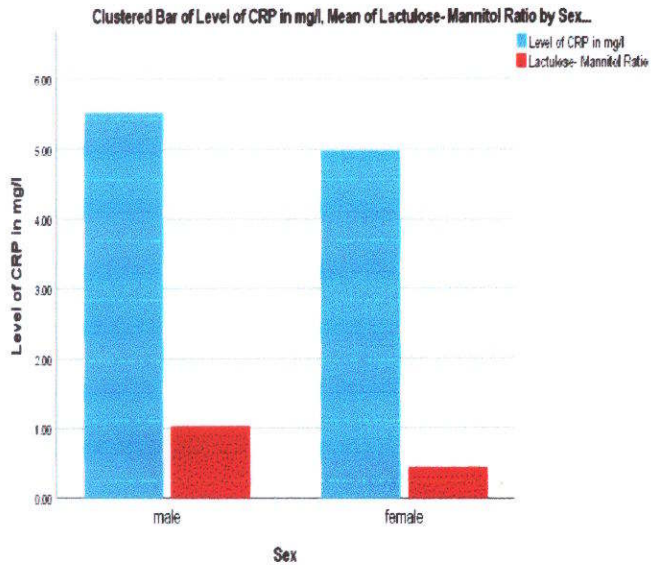
From above table 14 we could summarize that mean of L:M ratio between two nutritional status at risk of stunting and healthy group had nearly same mean difference as they had mean value .43 and 0.30 respectively which meant that gut barrier disruption in at risk group was little bit more than healthy group was present . Besides, HAZ (height for age z score) between at risk of stunting and healthy group had mean of HAZ -1.4 and -.34 respectively. In case of level of soluble CD14 between at risk of stunting and healthy group had high in stunted group bearing 1964ng/ml where at risk group taking mean 1948 ng/ml and from this we could say that in the blood level of soluble CD14 was high in the stunted group. Finally the level of inflammatory

biomarker CRP in at risk of stunting group had less than healthy group bearing mean 4.51mg/l and 5.80mg/l the reason for more amount of acute phase protein in the blood in healthy group may be others source inflammation which will be investigated in future.



29(a)





29(b) **29(c)**

Figure 29: Level of three non-invasive biomarkers among male and female

From this above figure 29 we described that level of soluble CD14 were high among female but level of CRP and L:M ratio were high in male.

Chapter Five: Discussion

Critical to the design and assessment of interventions for enteropathy and its developmental consequences in children living in impoverished conditions are non-invasive biomarkers that can detect intestinal damage and predict its effects on growth and development. I therefore, assessed fecal, urinary and systemic biomarkers of enteropathy and growth predictors in 233 infants of aged 52 weeks with varying degrees of malnutrition (stunting) in Mirpur, Dhaka, Bangladesh.

Environmental Enteric Dysfunction (EED) as assessed by elevated L: M was observed in over 65 per cent of participants in 52 weeks aged children from Mirpur, Dhaka, Bangladesh. Contrary to expectations, a biomarker of serum and stool intestinal health biomarkers demonstrated low agreement internally and with L: M ratio.

Taken together, the results suggest the biomarkers suffered from high variability and lower than expected associations with L:M ratio, raising questions in part about the ability of either L:M or a carefully selected set of serum and stool biomarkers to reliably identify cases of EED.

The prevalence observed for EED based on elevated L: M ratio is consistent with results from other research in similar settings and age groups. For example, in an urban Bangla desh cohort ages 6-24 months, the prevalence of elevated L: M ratio, using the 0.07 cutoff, was estimated at 71% in the non-malnourished reference population(7, 73),whereas in urban Nepal, 92% of children ages 0-60 months had elevated L: M. 92% of children 0-60 months of age had elevated L: M levels, using a cutoff of 0.12.(16, 21, 103).Studies in Gambia have consistently reported elevated L: M rates near 100% using the 0.12 cutoff in children under 5 years of age, (17, 91) whereas some studies in South Africa and Latin America found significantly lower EED rates.(13, 14, 88) Objectively high in prevalence, our observed EED burden is somewhat lower than in several other studies in South Asia, although values may be expected to rise up to 2 years of age before slowly returning to normal values during childhood.(74, 104)It should be noted that handling of L: M data, such as whether the recorded value was adjusted based on the administered dose, (39, 105)may all affect the observed prevalence of EED(106). Aside from those methodologic considerations, a somewhat lower burden of EED in this rural Bangladesh context is still plausible(107), as access to improved sanitation facilities and relatively clean tube well drinking water are common, in contrast to many other LMIC contexts(108, 109). Still, some sanitation and hygiene limitations persist, which have been associated with EED in a prior study in sever al districts of Bangladesh,(22, 69)And the burden of diarrhea(110) and the level of pathogenic and toxin exposures in this environment are all quite high.(2, 71)

It should also be noted that the parent trial could have had a positive impact on the risk of EED by enhancing nutritional status(19, 56) and, even in the control arm, by offering child feeding advice on hand washing, soap use and hygiene of food handling, as well as adequate hygiene.(65, 111)

It is also worth highlighting that while inflammation was common in this sample, neither CRP was closely related with L:M ratio nor Scd14. This is in contrast to hypotheses linking EED to growth by way of systemic inflammation,(2, 112)and suggests that other causes of systemic inflammation may be more prominent in this population (113). The primary cause(s) of elevated CRP(114) and sCD14 in this sample requires further investigation in and of itself, (43)and the lack of association between EED(115) and the acute phase proteins must be considered when linking EED to growth in this setting(110).

From soluble CD14 biomarker we could say that enteric dysfunction among study population close to 85% which was the most prevalence among of these three biomarkers whereas Stool biomarkers CRP results showed that over 20% infants had EED.

From my study I found that the percentage of stunted new born was declined as my study population data revealed that the stunted infants were over 23% which was decreased from previous study results 40% (45) the reason behind its improved sanitation and good hygiene practises.

Beside these, from my study data I had found that all of my non-invasive biomarkers value negatively correlated with HAZ score as a result we could say that the elevation of these biomarkers value correlates the stunted population number. Similar study they found that from 6 weeks to 12 months of age, levels of CRP and AGP were consistently higher and IGF-1 and IGFBP3 lower in cases versus controls but contradictory result with soluble CD14 as they did not find any association with soluabeCD14 with infant stunting (46). In my study, level of soluble CD14, CRP and L:M ratio had a negative correlation with HAZ score where (r= -.015, p=0.822), (r=-.028, p=.666).and (r=-.138, p=0.35) respectively.

The biomarker may have differed from L:M ratio because of differences in their underlying biology, but a lack of precision in the measurement of the set of biomarkers, indicated by large standard deviations relative to mean values, likely also contributed (110).

Some recent studies have questioned the validity of the L:M test, in particular suggesting that mannitol may be an inappropriate sugar for this purpose, (116)based on prior evidence that it can be found in the urine of un-dosed, fasting patients,(107, 117) and that an alternate analytic method to HPIC is necessary to accurately determine lactulose concentration (118).

Furthermore, the L:M ratio is thought to measure only two main aspects of the intestinal pathology characteristic of EED, increased permeability (70) and reduced absorptive capacity(20, 65), while the panel of serum and stool biomarkers (107)was selected deliberately to include markers covering a broader spectrum of EED effects (19, 118). Thus, some portion of the low agreement between the markers and L:M ratio may be due, by design to some extent, to their capturing different underlying processes(67). Unfortunately, without a better gold standard diagnostic test for EED, it is not possible to determine the extent to which other markers are providing additional information about EED status where they diverge from L:M ratio.

The present study had several strengths including the biomarkers that were assessed in combination with L:M ratio. Further, we were able to obtain and analyse biospecimens from an unusually large sample of children. The setting of the study nested within a large randomized trial also allowed for enrollment of participants from an enumerated source population, which, along with the very high participation rate, suggests a low risk of bias and good generalizability to urban Bangladesh and perhaps even more broadly to other rural South Asian populations.

Some weaknesses include that biospecimens were collected at one time point only, a trade off we accepted for a larger sample size, with the idea that more observations at a single time point would provide more data about the interrelationships and agreement among the set of biomarkers. We were also limited in the lack of appropriate normative data for several of the serum and stool biomarkers, as much remains unknown about their characteristics and performance under various normal conditions, a gap in knowledge that carefully conducted clinical studies may be most efficient in addressing. The main weakness that must be mentioned is the lack of a reliable gold standard measure of EED, a current limitation in this area of study more generally. In the absence of a true gold standard appropriate for this type of field setting, it is not possible to fully characterize other biomarkers in terms of their diagnostic properties.

Chapter Six: Conclusion

EED is a poorly defined, widely studied set of morphologic and functional changes to the small intestine. Maintaining adequate nutritional status and supporting growth in childhood rely on the proper functioning of the intestinal wall, both in its absorptive and barrier capacities. The EED literature suggests that those functions are widely impaired in LMIC settings and may explain a large portion of the stunting observed there. Observational and intervention studies aimed at determining the causes and risk factors for EED and preventive or treatment strategies have abounded, but progress is limited by the lack of a unified case definition and validated diagnostic tools appropriate for field settings. While those methodologic constraints inhibit drawing conclusions across studies, accumulating evidence suggests environmental and nutritional causes, perhaps required in combination, underlie the loosely defined EED syndrome. The strength of reported associations between intestinal health markers and growth varies widely between studies, and at present it is not clear the extent to which that link exists and in what settings, if any, EED may explain a large portion of observed stunting. Stunting remains a major public health problem, with implications for the health of individuals and their offspring and for the development of human capital. Inadequate diets, repeated morbidity episodes, environmental toxins and inadequate health care and other factors are known to constrain growth throughout the fetal, infancy and early childhood periods. Environmental and nutritional factors that impair the functioning of the intestine may exacerbate the effects of known risk factors on the development and persistence of stunting. EED may be a missing link that explains the intractability of stunting and holds the key to developing more effective interventions for the prevention of stunting. Many questions remain, however, and a critical first step in advancing this field of study will be the development of improved diagnostic measures appropriate for use in field settings. At present, the relationship between EED and stunting, and in particular, the contributions of diet quality and nutritional status, are not well described. Research elucidating those issues may provide valuable insight into the burden of stunting in LMICs and strategies for more effectively supporting healthy early childhood growth.

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